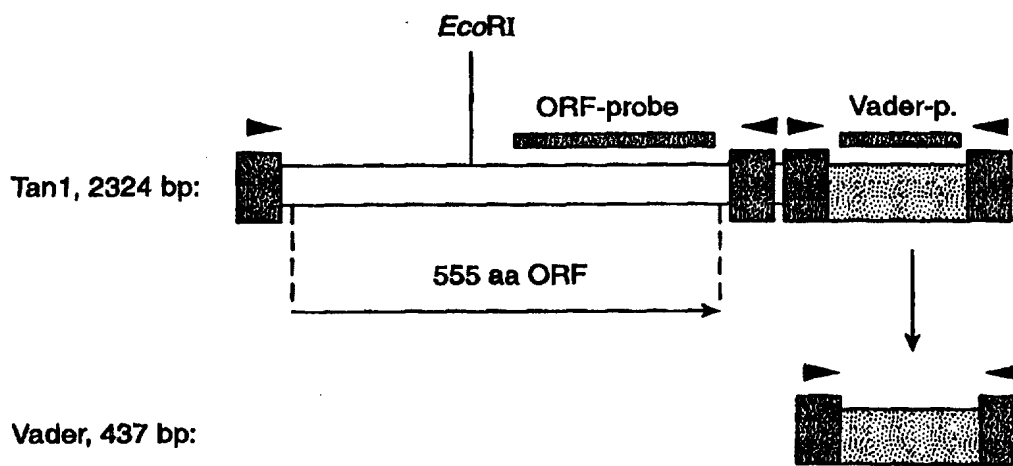




INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C12N 15/81, 9/22, C12Q 1/68, C12N 15/11	A1	(11) International Publication Number: WO 98/08960 (43) International Publication Date: 5 March 1998 (05.03.98)
(21) International Application Number: PCT/US97/14978 (22) International Filing Date: 25 August 1997 (25.08.97) (30) Priority Data: 08/703,077 26 August 1996 (26.08.96) US (71) Applicant: GENENCOR INTERNATIONAL, INC. [US/US]; 4 Cambridge Place, 1870 South Winton Road, Rochester, NY 14618 (US). (72) Inventors: AMUTAN, Maria; Genencor International, Inc., 925 Page Mill Road, Palo Alto, CA 94304-1013 (US). DUNN-COLEMAN, Nigel, S.; Genencor International, Inc., 925 Page Mill Road, Palo Alto, CA 94304-1013 (US). NYYSSONEN, Eini, M.; Genencor International, Inc., 925 Page Mill Road, Palo Alto, CA 94304-1013 (US). (74) Agent: GLAISTER, Debra, J.; Genencor International, Inc., 925 Page Mill Road, Palo Alto, CA 94304-1013 (US).		(81) Designated States: CA, JP, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>

(54) Title: IDENTIFICATION OF AND CLONING A MOBILE TRANSPOSON FROM *ASPERGILLUS*

**(57) Abstract**

There are provided transposable elements isolated from *Aspergillus*. Also provided are fragments comprising the inverted repeat(s) of the transposable elements, such fragments being useful as probes to isolate transposable elements from other filamentous fungi.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

IDENTIFICATION OF AND CLONING
A MOBILE TRANSPOSON FROM *ASPERGILLUS*

Field of the Invention

The present invention is directed at the identification, cloning and sequencing of mobile transposons or transposable elements from *Aspergillus niger* var. *awamori*. The transposable elements, referred to as Vader and Tan1, are approximately 437 base pair (bp) and 2.3 kb elements, respectively. The Vader and Tan1 elements are bounded by inverted repeat sequences of 44 and 45 base pairs, respectively. The transposable elements target a "TA" sequence in target DNA during insertion. In addition, the present invention is directed at the identification, cloning and sequencing of one or more transposable element(s) from other filamentous fungi using as a probe DNA comprising the Vader element 44 bp or the Tan1 element 45 bp inverted repeat isolated from *Aspergillus niger* var. *awamori*. Also provided are methods for utilizing either the Vader or Tan1 elements to inactivate genes (for example, by inserting the transposon into the gene to be inactivated), to overexpress a gene (by, for example, inserting a known promoter or other regulatory gene within the inverted repeats of Vader or Tan 1 and allowing the DNA of the IR-promoter-IR to jump in front of (and overexpress) a gene of interest) or to act as an activation marker to, for example, identify new promoters.

Background of the Invention

It is well known that transposons are a class of DNA sequences that can move from an episome to a chromosomal site or from one chromosomal site to another. Transposons are known in both prokaryotes, such as bacteria, as well as in eukaryotes, although there have been few transposons isolated from filamentous fungi.

Several groups have looked for transposons in filamentous fungi. The element *pogo*, which exists in multiple copies and at different sites in different strains of *Neurospora crassa*, was described by Schectman (1) and is believed to be a transposon. To date the most characterized transposon in filamentous fungi is Tad. Tad was isolated as a spontaneous mutant in the *am* (glutamate dehydrogenase) gene in an *Adiopodoume* strain of *N. crassa* isolated from the Ivory Coast. To detect mutations caused by insertion of a transposable element, Kinsey and Helber (2) isolated genomic DNA from 33 *am* mutant

-- 2 --

transposable element, Kinsey and Helber (2) isolated genomic DNA from 33 *am* mutant strains which were then screened by Southern analysis for restriction fragment size alterations. In two of the mutant strains, the mutation was shown to be caused by the insertion of a 7 kb element (Tad) into the *am* gene. Subsequently Kinsey (3) demonstrated that Tad was able to transpose between nuclei of heterokaryons, confirming that Tad was a retrotransposon and that there was a cytoplasmic phase involved in the retrotransposition events. More recently, Cambareri et al. (4) demonstrated that Tad was a LINE-like DNA element with two major open reading frames (ORFs) on the plus strand. Typical of LINE-like elements, Tad had no terminal repeats. Attempts to isolate mobile transposons in laboratory strains of *N. crassa* were unsuccessful.

A second retrotransposon was cloned by McHale et al. (5), who reported the isolation of CFT-1, an LTR-retrotransposon from *Cladosporium fulvum*. This transposon was 6968 bp in length and bounded by identical long terminal repeats of 427 bp, a 5 bp target site duplication. Virus-like particles were detected which co-sediment with reverse transcriptase activity in homogenates of this fungus.

Daboussi et al. (6) were the first to successfully use the *niaD* (nitrate reductase) gene as a transposon trap. The *niaD* mutants can be isolated by a direct selection for chlorate resistance (7). The strategy employed was to isolate *niaD* mutants amongst six isolates belonging to different races of the fungus *Fusarium oxysporum*. More than 100 *niaD* mutants were isolated from each isolate and examined for instability. One strain, F24, yielded up to 10% unstable *niaD* mutants. Assuming that the genetic instability of the *niaD* mutants was caused by transposable elements, it seemed plausible that this isolate contained mobile transposons. A stable *niaD* mutant in the F24 was transformed with the cloned *niaD* gene from *A. nidulans* because the *F. oxysporum niaD* gene had not been cloned. Unstable *niaD* mutants were isolated in transformants containing the *A. nidulans niaD* gene. Two unstable *niaD* mutants were shown by Southern blot analysis to contain an insertion of 1.9 kb in size. Analysis of this element, *Fot1*, revealed it was 1928 bp long, had a 44 bp inverted terminal repeats, contained a large open reading frame, and was flanked by a 2 bp (TA) target site duplication. Very recently, Daboussi et al. (8) have reported the cloning of a new transposable element from an unstable *niaD* mutant. This element, *FML* (*Fusarium mariner*-like), is 1280 bp long and has inverted repeats of 27 bp. The *FML* element inserts into a TA site and excises imprecisely.

Using the characterization of unstable *niaD* mutants strategy, Lebrun et al. (9) were able to isolate a transposon from *Magnaporthe grisea*. However, in this case the A.

-- 3 --

nidulans niaD gene which was transformed into *M. grisea* by transformation was used as a transposon trap. The element inserted into the *niaD* gene was shown to belong to a family of *M. grisea* LTR-retrotransposons, Fos1 (Schull and Hamer, unpublished) and Mag1 (Farman and Leong, unpublished). The cloned retro-element was 5.6 kb and the target site (ATATT) was shown to be duplicated. All revertants from this mutant examined had one copy of the LTR left at the point of insertion. A second transposon, Pot2, from *M. grisea* was recently cloned by Kachroo et al. (10). The strategy used to clone Pot2 was to analyze the fingerprint patterns of repetitive DNA's which were cloned from the *M. grisea* genome. A repetitive family present in both rice and non-rice pathogens of *M. grisea* in high copy number was cloned. The element, 1857 bp in size, has a 43 bp perfect terminal inverted repeats (TIR) and 16 bp direct repeats within the TIRs. An open reading frame was shown to display extensive identity to that of Fot1 of *F. oxysporum*. As with Fot1, the Pot2 element duplicates the dinucleotide TA at the target insertion site. Pot2 was shown to be present at a copy number of approximately 100 per haploid genome.

Several groups have reported looking without success for transposons in laboratory strains of *A. nidulans* (Kingham personnel communication, 5). One explanation for the lack of transposons in laboratory strains is that the desirable features of strain stability required for genetic analysis may preclude strains with mobile transposon. By using the *niaD* gene as a transposon trap we have identified and isolated a transposable element from the industrially important fungus *A. niger* var. *awamori*. This element, Vader, is present in approximately 15 copies in *A. niger* and *A. niger* var. *awamori*. Southern analysis of *A. nidulans* with this element indicates that this transposable element was absent from one laboratory strain and only present as a single copy in a second laboratory strain. These results support the notion that laboratory strains of *A. nidulans* contain very few transposons.

Brief Description of the Invention

In accordance with the present invention, novel eukaryotic transposable elements from *Aspergillus niger* var. *awamori* are provided. The larger transposable element, referred to herein as Tan1, is 2.3 kb in size. The smaller transposable element, referred to herein as Vader, is a 437 bp element (SEQ ID NO:3). Vader is found within the larger element Tan1. The Vader transposable element is a 437 bp element which comprises a 44 bp inverted repeat sequence at either end of the transposable element. Tan1 is approximately a 2325 bp element which comprises 45 bp inverted repeats at either terminus and internal IRs. Tan1 comprises a 555 aa open reading frame (ORF) which

-- 4 --

codes for a transposase which allows the elements (Tan1 or Vader) to "hop" or insert themselves in the genome of a host. The target for insertion of these novel transposable elements is a "TA" sequence in the target DNA for insertion. The "TA" sequence is repeated at either end of the transposon upon insertion of the transposable element into the target DNA. Therefore, the present invention provides the larger Tan1 transposable element as well as the smaller element (Vader) internal thereto, as well as the DNA encoding each.

Another embodiment of the present invention comprises a fragment of the Vader or Tan1 transposable elements which comprise the 44 or 45 bp (respectively) inverted repeat sequences found at either terminus of the transposable element from *A. niger* var. *awamori*, as well as the use of said fragments as probes to hybridize under low stringency conditions to DNA of other filamentous fungi for the isolating and/or cloning of transposable elements from such other filamentous fungi. While the exact 44 bp IR of Vader or the 45 bp IR of Tan1 can be utilized, it is well understood by those skilled in the art that variation of such DNA would also work as a suitable probe. For example, at a minimum, the imperfect direct repeats within the IRs of Tan1 would be suitable to use as probes for isolating transposable elements from other filamentous fungi. Initially the inverted repeat of Vader was used to clone Tan1 using PCR techniques. This work was followed by obtaining a genomic copy of Tan1 from a partial library.

Another embodiment of the present invention is the transposase activity coded for by the ORF of Tan1. This transposase is 555 aa (SEQ ID NOS:7 or 14, PCR and genomic, respectively).

In a process embodiment of the present invention there are provided methods for gene tagging comprising using the transposable elements of the present invention (Vader or Tan1 or any transposable element isolated using the IRs of either) to inactivate genes via insertion of the element into a given gene, thus disrupting or inactivating gene expression. Alternatively, the transposable element can be used in activation tagging (to activate or turn on genes) rather than for gene disruption. For example, by inserting DNA coding a promoter into the transposable element and then allowing such transposable element to become inserted 5' to a desired gene, the promoter may be activated to drive the expression of the desired gene product or to turn on cryptic pathways. Additionally, gene tagging can be utilized to activate marker genes by inserting a marker gene within the IRs of a transposon of the present invention. This marker gene can then "hop" into targeted DNA and, if expression of the marker is selected for, it will be possible to identify

the promoter driving such expression. This may lead to identification of isolation of new strong promoters.

Brief Description of the Drawings

5 Fig. 1 shows the Southern blot analysis of unstable *niaD* mutants. PCR-amplified genomic *niaD* gene from four *niaD* mutants and UVK143f were digested with BglII (sites are 3' of all inserts). Blot probed with 500 bp fragment of Sall digested PCR product of *niaD*1 and *niaD*2. Wild-type band hybridizes at 2.5 kb while gene with insertion hybridizes at 2.9 kb. Lanes: 1=MW marker III (Boehringer Mannheim); 2=UVK143f; 3=*niaD*410; 4=*niaD*436;
10 5=*niaD* 587; 6=*niaD*392.

Fig. 2 depicts the mapping of Vader insertions within the *niaD* gene. The positions of Vader insertions 1-4 (*niaD*410, *niaD*436, *niaD*587 and *niaD*392, respectively) are shown relative to the six introns of the structural gene coding region. Because the exact site of insertion for Vader-1 and Vader-4 is still unknown, they have been presented using the
15 approximate area of insertion. Relevant restriction sites are shown using the following letters: E=EcoRI, S=Sall, Sp=SpHI, K=KpnI, and B=BglII.

Fig. 3 shows Southern blot analysis to determine Vader genomic copy number. Four *A. niger* var. *awamori* *niaD* mutants and UVK143f were digested with EcoRV to completion. EcoRV cuts the Vader sequence once. Hybridization indicates that Vader is
20 present in the genome in more than 14 copies. The hybridizing bands of *niaD* 392, which are different from the other mutants and UVK143f, suggest that the Vader sequence is mobile. Lanes: 1=MW marker III, 2=UVK143f, 3=*niaD*410, 4=*niaD* 436, 5=*niaD* 587, 6=*niaD* 392.

Fig. 4. Southern blot to determine presence of Vader sequence in other fungi. Other filamentous fungi, an industrial production strain and *niaD* mutant 392 were digested
25 with EcoRV to completion. Low stringency hybridization (32) indicates that sequences homologous to Vader are present in *A. nidulans* (FGSC A237), *A. cinamomeus*, *A. phoenicis*, *A. foetidus*, an industrial *A. niger* strain. Lanes: 1=MW marker, 2=*A. foetidus*, 3=an industrial glucoamylase production strain of *A. niger* (ETC #2663), 4=*A. niger* var. *awamori* *niaD* mutant 392, 5=*A. phoenicis* (ATCC #11362), 6=*A. nidulans* (FGSC A691),
30 7=*A. wentii* (ATCC #10593), 8=*A. versicolor*, 9= *A. cinamomeus* (ATCC #1027), 10=*A. nidulans* (FGSC A237)

Fig. 5. Southern blot to determine Tan1 (transposon from *A. niger*) genomic copy number. Four *niaD* mutants *A. niger* var. *awamori* mutants and UVK143f were digested

with *EcoRI* to completion. *EcoRI* cuts the Tan1 sequence once. A probe corresponding to the ORF region (see Fig. 9) was used in the hybridization. Hybridization indicates that Tan1 is present as a single copy in the genome. Lanes: 1=MW marker III, 2=UVK143f, 3=*niaD*410, 4=*niaD* 436, 5=*niaD* 587, 6=*niaD* 392.

5 Figs. 6A-6C. Southern blots to determine if the inverted repeats of transposable elements Fot1 and Pot2 will hybridize to elements in *A. niger* var. *awamori*. Four *niaD* mutants *A. niger* var. *awamori* mutants were digested with *EcoRI* to completion. *EcoRI* cuts the Tan1 sequence once. Inverted repeat oligonucleotide probes of Vader (SEQ ID NO:5), Fot1 and Pot2 were labeled with digoxigenin (Boehringer Mannheim). Lanes: 1=MW
10 marker III, 2=*niaD*436, 3=*niaD*587. Blot A (lanes 1-3) and B and C were probed with the labeled inverted repeat probes of Vader, Fot1 and Pot2, respectively.

Fig. 7 shows the sequence of the Vader insertion (SEQ ID NO:3) as generated by PCR. Vader was found to be 437 bp in length. The 44 bp inverted repeat of the Vader insert corresponding to SEQ ID NO:4 (the 5' IR) and SEQ ID NO:5 (the 3' IR), respectively,
15 from the 5' end to the 3' end of Vader are underlined, the single mismatch which occurs in the inverted repeats is identified in bold, and the TA 2 bp duplication is shown in bold print. *niaD* sequences flanking the element are shown in lower case letters.

Figs. 8A and 8D show the entire DNA sequence of the Tan1 element (SEQ ID NO:6) as generated by PCR, as well as the putative amino acid sequence of the
20 transposase coded for by Tan1 (SEQ ID NO:7). Tan1 as generated by PCR is 2320 bp in length (excluding the unknown nucleotides shown as "N" in the figure) and has a large open reading frame of 1668 bp which encodes for 555 amino acids (SEQ ID NO:7). Tan1 comprises the sequences of four inverted repeats (underlined) similar to those found in Vader.

25 Fig. 9 shows a schematic presentation of Vader and Tan1 elements. Dark boxes represent the 45 bp (Tan1) and 44 bp (Vader) inverted repeats. The unique *EcoRI* site in the Tan1 element was used for digestion of genomic DNA in Southern analysis (Figs. 5 and 10). Bold, horizontal lines above the Tan1 element indicate the probes corresponding to the end of the ORF and Vader used in Southern analysis shown in Fig. 10 and Fig. 5.

30 Fig. 10 shows Southern analysis of *A. niger* var. *awamori niaD* mutants (*niaD*410, *niaD*436, *niaD*587, *niaD*392) and the wild-type UVK143f: lane 1, molecular weight marker III (Boehringer Mannheim); lane 2, UVK143f; lane 3, *niaD*410; lane 4, *niaD*436; lane 5, *niaD*587; lane 6, *niaD*392. This blot was probed for the Vader element (see Fig. 9). When this blot (Fig. 10) was superimposed with the blot shown in Fig. 5, one of the illuminated
35 bands from the Vader-probe hybridization overlaid the single band in the ORF-probe

hybridization indicating that the Tan1 element is composed of contiguous ORF and Vader elements.

Figs. 11A and 11D show the nucleotide sequence (genomic copy) of Tan1 (SEQ ID NO:13). The amino acid sequence encoding the putative transposase (555 aa) (SEQ ID NO:14) is shown below the DNA sequence in the one-letter amino acid code. The inverted repeats are underlined (SEQ ID NOS:1, 2, 15 and 16, respectively, 5' to 3') and the imperfect direct repeats within the inverted repeats are shown with arrows above or below the sequence. The gaps within the arrows indicate the imperfect nucleotides within the direct repeats. Undetermined sequence is denoted in the figure by question marks and in the sequence listing as "N." The figure shows the DNA sequence as 2324 base pairs, excluding the unknown nucleotides indicated by "?" in the figure.

Detailed Description of the Invention

While the specification concludes with claims particularly pointing out and distinctly claiming the subject matter regarded as forming the present invention, it is believed that the invention will be better understood from the following detailed description of preferred embodiments.

Standard biochemical nomenclature is used herein in which the nucleotide bases are designated as adenine (A); thymine (T); guanine (G) ; and cytosine (C). N connotes any of these nucleotides. As is conventional for convenience in the structural representation of a DNA nucleotide sequence, only one strand is usually shown in which A on one strand connotes T on its complement and G connotes C.

Applicants have isolated two transposable elements from *A. niger* var. *awamori*. The cloned element Vader was identified by screening unstable nitrate reductase (*niaD*) mutants for insertion. This element is present in approximately fifteen copies in the genome of *A. niger* strains examined. In contrast, the Vader element is present in one copy in only one of the two *A. nidulans* strains studied. These results explain why several groups have been unsuccessful in isolating active transposons in laboratory *A. nidulans* strains. A plausible assumption is that "domesticated" strains of *A. nidulans* have lost their transposons due to repeated manipulation of such strains and the possible discarding of aberrant *A. nidulans* strains displaying genetic instability.

The Vader element shows similarities to transposable elements cloned from the plant pathogens Pot1 from *M. grisea* (12) and Fot1 from *F. oxysporum* (8). The target site for duplication in all three fungi is a 2 bp TA sequence. In the case of Fot1, this transposon does not excise precisely. In two *niaD* revertants examined, the excision products retained a 4 bp insertion relative to the wild-type gene (TAATTA versus TA). The insertion studied

was integrated into an intron, therefore, imprecise excision of Fot1 did not effect the functionality of the *niaD* gene product. There is no published evidence that Pot2 is a functional element.

A homology search made at the nucleotide level gave a strong 60.7% homology
5 between Tan1 and a 1230 bp overlap to the *A. oryzae agdA* gene coding for an α -glucosidase (33). This homology search revealed that the last 1.2 kb of a total of 5.2 kb of the α -glucosidase sequence submitted to GenBank is, in fact, part of a novel transposon, hereinafter called *Tao1* (transposon *Aspergillus oryzae*), which also belongs to the *Fot1* family. Only the 5' half of the *Tao1* element is included in the GenBank sequence, thus, for
10 the lack of comparison, the exact size of the inverted repeat cannot be determined. However, it can be concluded that there are 13 bp perfect direct repeats within the inverted repeat. The inverted repeat is flanked by a TA-dinucleotide, suggesting a commonly occurring TA-insertion site. Direct analyses gave only short ORFs, but when the often-occurring stop codons were ignored, a long ORF was obtained which shared over 50%
15 identity to the *Tan1* transposase. Multiple stop codons indicate that the *A. oryzae Tao1* is a defective element. This transposable element from *A. oryzae*, thus, is within the scope of the present invention as, based on the high degree of sequence homology between the Tan1 and *Tao1*, it is believed that *Tao1* would hybridize to a probe comprising Tan1 or Vader IRs or variations thereof. The sequence of the IR of *Tao1* is provided as SEQ ID
20 NO:17. This IR (*Tao1*) or the IRs from Tan1 or Vader may be used to isolate other transposable elements from filamentous fungi.

In an attempt to determine if there were transposons similar to those reported for *F. oxysporum* and *M. grisea*, synthetic oligomers were made corresponding to the inverted repeats of both Fot1 (7) and Pot2 (10). When Southern analysis of *A. niger* var. *awamori*
25 was conducted using the Vader 44 bp inverted repeat (SEQ ID NO:5) as a control, no conclusive hybridizations could be detected with either the Fot1 or Pot2 oligomeric probe. These results indicate that elements with high identity to *F. oxysporum* Fot1 and *M. grisea* Pot2 are not found from *A. niger* var. *awamori* genome.

With regard to the structure of the Vader element, elements which transpose
30 directly through DNA copies are typified by having inverted terminal repeats. Elements which transpose through reinsertion of the product of reverse transcription of an RNA copy of the element (retroelements) can be without long terminal repeats such as the *Drosophila* I element (for a review see (16)). Alternatively, retrotransposons can have long terminal repeats such as the *Drosophila copia* element. The Vader inverted repeats shown in Fig. 7,

-- 9 --

SEQ ID NOS:4 and 5, respectively, have a single mismatch. Elements which transpose through DNA copies typically have open reading frame(s) which encode a transposase activity. The Fot1 element is 1.9 kb in length and the Pot1 element 1.8 kb in length. Both the Fot1 and Pot1 elements have ORF encoding for a putative transposase-like protein.

5 The Vader element, although mobile, does not have an ORF and hence it was deduced that the mobility of Vader was dependent upon a transposase activity present elsewhere in the genome. A synthetic 44 bp oligomer of the inverted repeat of Vader (SEQ ID NO:5) was used to clone, via PCR, a 2.3 kb element. This element, called Tan1 (SEQ ID NO:6), comprises four inverted repeats (SEQ ID NOS:1, 2, 15 and 16 from 5' to 3', respectively)
10 similar to those in Vader and has a unique organization IR-ORF-IR-IR-Vader-IR. Tan1 is 2324 bp in length and has a large open reading frame (1668 bp) which encodes a putative transposase comprising 555 amino acids (shown in SEQ ID NOS:7 and 14), which is homologous to Fot1 and Pot2 transposases. Immediately 3' to the second IR (SEQ ID NO:2), which bounds the transposase, is a copy of the Vader element. We hypothesize
15 that at some stage the independent Vader element, although inactive by itself, has arisen from Tan1, resulting in current strains with only one copy of Tan1 providing transposase activity and numerous mobile copies of Vader dispersed in the genome.

Thus, applicants have been the first to identify a transposable element(s) with certain *Aspergillus* species. These transposable elements are believed to be quite useful in
20 the development of gene tagging systems for *Aspergillus* or other microorganisms. Basic requirements for developing a gene tagging system are that the tagging element can be distinguished from the endogenous elements, it displays little sequence specificity for transposition and that excision is followed by integration at a new site. More refined tagging systems include ability to monitor excision and reinsertion by, e.g., activation of
25 antibiotic resistance genes and ability to stabilize the mutations by, e.g., a two transposons system (23, 24 and 25).

For development of a tagging system for *Aspergillus*, it is proposed that the system is tested first in *A. nidulans*, which we have already shown does not have endogenous Tan1 or Vader sequences. However, at this stage the Vader element is altered from the
30 original in such a way that the same construction can be later used in *A. niger* var. *awamori* and be distinguished from the endogenous Vader elements.

In a model tagging system using Vader as the "mutator," a first vector can be constructed for expression of the Vader element, similar to the non-autonomous maize Dc. The internal sequence of the Vader element is altered to contain translation initiation and
35 stop codons in three different frames. This sequence can later be used as a recognition

site for a probe in PCR analysis of the mutants. This altered Vader element, Vader-S, is inserted within an expression cassette conferring antibiotic resistance such as hygromycin resistance. Since excision of Vader may not always be precise, Vader-S is inserted in the promoter area (e.g., *oliC*) between the transcription and translation initiation sites. This
5 disrupted hygromycin phosphotransferase cassette is flanked by marker genes - or alternatively the marker gene upstream of the hygromycin promoter can be placed within Vader. These marker genes can be used for monitoring whether the hygromycin gene, and Vader within it, have integrated in full length. A vector, for example, Vector I, containing these elements will be transferred to *A. nidulans* and transformants expressing the two
10 marker genes, but sensitive to hygromycin, are selected. Screening of mutants at later stages is easier, if the transformant selected for mutagenesis has only one to two copies of Vector I sequences integrated in its genome.

A transformant with only a few (preferentially one) intact Vader-S/hygromycin phosphotransferase cassettes integrated in its genome is retransformed with Vector II,
15 which is an autonomously replicating vector carrying the transposase encoding gene. The autonomously replicating vector, pHELP, used as a basis for DNA construction work, can be segregated away by methods known to those skilled in the art. This enables stabilization of the Vader-S element after the mutagenesis step. Vader-S is activated by a transposase (from Tan1) in pHELP, which can be monitored by activation of the
20 hygromycin resistance gene. Tan1 is not cloned into the vector in full length to disrupt its mobility. Again, Vector II contains a marker gene used for screening of transformants and also for monitoring its segregation after the sporulation phase.

Marker genes can either complement host mutations or be dominant markers such as benomyl^R, acetamidase or β -glucuronidase (GUS).

25 In a model system for gene tagging the target gene for mutagenesis should be one with a simple plate screen, e.g., disruption of the *niaD* gene (by insertion of Vader), which can be screened by selection of chlorate resistant mutants and the gene disruption can be further mapped by a plate test using different nitrogen sources (no growth on nitrate, growth on nitrite, xanthine and uric acid). Another target gene for mutagenesis could be an
30 acid protease gene. It has been shown previously for *A. niger* that disruption of this one protease is sufficient to abolish halo formation almost completely on skim milk plates.

The advantage of using transposon tagging is that the mutants produced can be identified by subsequent isolation of the mutated gene. There are several methods available for PCR amplification of genomic sequences when only one end of the sequence
35 is known - which, in this case, is the transposable element. PCR methods developed for

-- 11 --

genomic walking are, e.g., "Inverse PCR" (27 and 28), "Vectorette PCR" (29) and "Panhandle PCR" (30).

Setting up the transposon tagging system can be followed by studies of excision frequency, environmental influences on transposition frequency (24, 31), activation of the transposase by a heterologous promoter and effect of altered inverted repeats on transposition.

Transposon tagging does need to be applied for inactivation of genes. Alternatively, tagging can be used to insert promoter sequences in *Vader* and therein activate genes. A third option is to insert a promoterless marker gene in *Vader*, in which case the transposon can be used in search for novel, strong fungal promoters.

Experimental

Materials and Methods

Strains. *Vader* and Tan1 elements were isolated from *Aspergillus niger* var. *awamori* UVK143f, derived from Northern Regional Research Laboratories (NRRL) #3112. *E. coli* JM101 [*F'* *traD36 lac1^q Δ(lacZ)M15 proA⁺B⁺ /supE thi Δ(lac-proAB)*] and *Epicurian coli* SURE 2 (Stratagene Cloning Systems, La Jolla, CA) were used for propagation of *Vader* and Tan1 subclones, respectively.

Spontaneous chlorate resistant mutants were derived from *Aspergillus niger* var. *awamori* UVK143f (NRRL #3112). The following *Aspergillus* strains were obtained from the ATCC: *A. cinnamomeus* (ATCC #1027), *A. wentii* (ATCC #10593), and *A. phoenicis* (ATCC #11362). *A. nidulans* (FGSC #A237), a nitrate reductase structural gene mutant (*niaD15*), and *A. nidulans* (FGSC #A691), a tryptophan requiring mutant (*trpC801*), were obtained from Fungal Genetics Stock Center (FGSC), Dept. of Microbiology, University of Kansas Medical Center. *A. versicolor*, *A. foetidus*, and a proprietary *A. niger* glucoamylase strain are from the Genencor International Inc. culture collection.

Mutant Selection. Spore suspensions (1×10^8) of UVK143f were plated on CM agar (11) containing 600 mM KClO_3 and 10 mM glutamic acid. Chlorate (KClO_3), a toxic analog of nitrate, allows selection of mutants in the nitrate assimilation pathway by chlorate resistance. Plates were incubated at 37°C until individual colonies of spontaneous mutants could be identified. Single mutants resistant to KClO_3 were allowed to sporulate on CM plates and spores from these plates were then streaked onto minimal media (11) with various sole nitrogen sources (10 mM): NaNO_3 (nitrate), NaNO_2 (nitrite), hypoxanthine, uric acid or NH_4Cl (ammonium chloride). Each of these compounds are intermediate products

-- 12 --

of the nitrate assimilation pathway. *niaD* mutants were identified as those resistant to KClO_3 and able to grow in the presence of all pathway intermediates, except for NaNO_3 .

Isolation of Vader via PCR Amplification. Genomic DNA of *A. niger* var. *awamori* *niaD* mutants and UVK143f was used as template (see Southern Analysis). Primers (50 pmol) used for amplification of the *niaD* gene were NiaD1 (position 142-165 relative to the initiation site of *niaD*): 5'-CCAACCGAGTCCTCAGTATAGAC-3' (SEQ ID NO:8) and NiaD2 (position 2738-2715): 5'-CAACGCTTCATAGGCGTCCAGATC-3' (SEQ ID NO:9). Deep Vent (exo⁻) DNA polymerase (New England Biolabs) was used with the buffer and dNTPs provided by the manufacturer. For optimal amplification of the *niaD* gene the reaction mixture contained 4 mM MgSO_4 . Denaturation of template DNA, 2 min. at 94°C, was followed by 30 cycles of denaturation (30 sec. at 94°C), annealing of primers (45 sec. at 55°C) and extension (4 min. at 72°C). PCR fragments were purified from gel using the Qiaex DNA gel extraction kit (Qiagen), digested and used for restriction enzyme analysis by standard procedures (12).

Confirmation of Excision Foot Print by PCR Amplification and Sequencing.

Template DNA from *niaD*436 was used in a PCR reaction in an attempt to amplify both the larger *niaD* sequence with an insert and the shorter *niaD* fragment resulting from excision of the Vader element. The PCR reaction was conducted as previously described, except for using primers MA003 (positions 359-378): 5'-ATATGAATTCCTTCTTGACTTCCCCGGAAC-3' (SEQ ID NO:11) and NiaD5 (position 1125-1144): 5'-ATATAAGCTTGTCACTGGACGACATTTTCAG-3' (SEQ ID NO:12). The gel purified fragment (ca. 800 bp) resulting from the excision event was submitted for sequencing.

Isolation of Tan1 via PCR Amplification. Fungal genomic DNA for PCR and Southern analyses was isolated from mycelia grown in CSL supplemented with 5% fructose (21). Genomic DNA of *A. niger* var. *awamori* *niaD* 436 mutant (22) was used as a template. A single primer (100 pmol), IR1, was used for amplification of Tan1. The 54-mer IR1 was derived from the 44 bp inverted repeat sequence of Vader preceded by a restriction enzyme recognition site for *Eco*RI: 5'-ATATGAATTC ACGTAATCAA CGGTCGGACG GGCCACACGG TCAGGCGGGC CATC-3' (SEQ ID NO:10). Deep Vent (exo⁻) DNA polymerase (New England Biolabs) was used with the buffer and dNTPs provided by the manufacturer. Denaturation of template DNA, 10 min. at 94°C, was followed by 30 cycles of denaturation (1 min. at 94°C), annealing of primers (1 min. at 55°C) and extension (6 min. at 72°C). PCR fragments were purified from agarose gels

-- 13 --

using the Qiaex DNA gel extraction kit (Qiagen) and subcloned as blunt-ended inserts into *EcoRV* cut pSL1180 (Pharmacia Biotech).

Estimation of *niaD* Mutant Reversion Frequency. Spores from *niaD* mutants *niaD392*, *niaD410*, *niaD436* and *niaD587* were streaked onto minimal media containing NaNO₃ as a sole nitrogen source. Nitrate non-utilizing colonies of *niaD* mutants, which had a spidery appearance and did not sporulate, were streaked onto CM containing 600 mM potassium chlorate (KClO₃) and incubated to confluency at 37°C. Ten-fold dilution series of spore suspensions (in 0.8% NaCl-0.25% Tween 80) of *niaD392*, *niaD410*, *niaD436*, *niaD587* and UVK143f wild-type spores were plated on minimal media with nitrate (10 mM) to determine reversion frequency, and on CM to determine viability.

Southern Analysis. Genomic DNA for PCR and Southern analysis was isolated (13) from mycelia grown in CSL (13), which contained 600 mM KClO₃ in order to reduce reversion of *niaD* back to the wild-type during cultivation. DNA (10 µg) was digested with either *Bgl*III, which leaves the insertion intact in the *niaD* gene, or with *EcoRV*, which cuts the insertion element (Vader) once, and thus enables determination of its copy number in the genome. Genomic DNA (approximately 10 µg) of *A. nidulans*, *A. cinnamomeus*, *A. versicolor*, *A. wentii*, *A. phoenicis*, *A. foetidus* and of an industrial *A. niger* strain were digested with *EcoRV* to obtain an estimate of Vader copy number in these fungal genomes. The digested and gel-separated DNA was transferred to a positively-charged nylon membrane (Boehringer Mannheim) by capillary action.

The DNA probe for the *niaD* gene was derived from the PCR product (UVK143f DNA template amplified with primers NiaD1 (SEQ ID NO:8) and NiaD2 (SEQ ID NO:9)), which was digested with *Sall*, resulting in a 528 bp probe fragment. The probe for the insertion element, Vader, was derived from a PCR reaction in which *niaD436* DNA was used as a template. This PCR product was purified and digested with *Sall* and *Sph*I and subcloned into the vector pUC19. This subclone was digested with *Scal* and *Xba*I to yield a 236 bp fragment which was used for estimation of the copy number of Vader sequences in the genomes of various fungi.

A DNA labeling and detection kit (Genius1, Boehringer Mannheim) was used for random primed labeling of probe DNA with digoxigenin, and for detection with alkaline-phosphatase labeled antibody to digoxigenin.

Hybridization and washing conditions for homologous probes were conducted as recommended by the manufacturer using hybridization buffer without formamide at 68°C (Boehringer Mannheim). Hybridizations for heterologous Southern analysis (i.e., analysis

of DNA from other *Aspergillus* sp.) was conducted using hybridization buffer with 25% formamide at 37°C. Washes were performed as in stringent wash protocol.

Nitrate Reductase Assays. Nitrate reductase assays were performed as described in Dunn-Coleman, et al. (18).

5 **DNA Analysis and Sequence Determination.** Sequences were determined using fluorescent-labeled dideoxynucleotide terminators and *Taq* cycle sequencing on the 373A sequencer (ABI). Commercially available universal and reverse (New England Biolabs) primers were used. Alignment of sequences and prediction of amino acid sequences were performed using DNASTAR (DNASTAR, Inc.). The nucleotide and deduced amino acid
10 sequences were analyzed and compared to those in GenBank, EMBL and Prot-Swiss using Fast A and BLAST programs (Genetics Computer Group, Inc. software package, Madison, WI).

15 **Other Probes Used for Southern Analysis.** The Tan1 probe was prepared by digesting Tan1 with *HindIII* and *StuI* resulting in a 650 bp fragment corresponding to the 3' end of the transposase coding region (ORF-probe in Fig. 9). The Vader element was digested with *XbaI* and *ScaI* to yield a 236 bp fragment to be used for recognition of internal Vader sequence in Southern analysis (Vader-probe in Fig. 9).

20 **Southern Analysis to Determine Tan1 Copy Number.** *Aspergillus* genomic DNA (10 µg) was digested with *EcoRI*, which cuts the Tan1 element once in the transposase coding region and upstream of sequences corresponding to the Vader and Tan1 probes used in hybridizations (Figs. 5, 9 and 10). DNA labeling and detection kit (Genius 1, Boehringer Mannheim) was used for random primed labeling of probe DNA with digoxigenin and for detection with alkaline-phosphatase labeled antibody to digoxigenin. Hybridization and washing conditions were conducted as recommended by the
25 manufacturer (Boehringer Mannheim).

30 **Isolation of Tan1 from a Partial Genomic Library.** It was known from the sequence of the PCR-amplified Tan1 element that Tan1 did not have restriction enzyme recognition sites for *BglII* and *XhoI*. A *BglII*-*XhoI* digested Southern blot of *Aspergillus niger* var. *awamori* genomic DNA, hybridized with the 650 bp *HindIII*-*StuI* Tan1 probe, resulted in identification of a 4.5 kb genomic fragment containing Tan1. *A. niger* var. *awamori* *niaD436* DNA was digested with *BglII* and *XhoI* and fragments in a size range of 4-5 kb were cloned into pSP73 (Promega). This partial genomic library was screened by colony hybridization using the nonradioactive nucleic acid labeling and detection system from Boehringer Mannheim.

-- 15 --

Example 1**Isolation of Spontaneous High Frequency Reverting
niaD Mutants of *A. niger* var. *awamori***

Assuming that *niaD* mutants which arise from the insertion of a transposable
 5 element would be unstable, a total of 152 *niaD* mutants, isolated on the basis of
 spontaneous resistance to chlorate were characterized. To determine if the *niaD* mutation
 was unstable, spores from 43 *niaD* mutants were plated onto medium with nitrate as the
 sole nitrogen source. Fourteen of the mutants reverted to the wild-type phenotype at a
 frequency of greater than 1×10^{-5} . Table 1 summarizes the *niaD* mutant reversion studies.

Table 1

<u>Mutant</u>	<u>Conidia Plated No. x 10³</u>	<u>No. Wild-Type Colonies</u>	<u>Reversion Frequency x 10⁻⁴</u>
15 <i>niaD</i> 392	2.9	27	93
<i>niaD</i> 410	7.7	5	6.5
<i>niaD</i> 436	3.7	164	443
<i>niaD</i> 587	18.9	12	6.3

There appeared to be two classes of *niaD* mutants which reverted at high
 20 frequency. The *niaD* mutants *niaD*436 and *niaD*392 reverted at high frequency, while
 mutants *niaD*410 and *niaD*587 yielded smaller numbers of revertant colonies.

The level of nitrate reductase activity was determined using the assay described in
 (18) from revertant colonies isolated from the *niaD* 436 mutant. Nitrate reductase activity
 was detected in 14 of 15 revertants analyzed (see Table 2). A spectrum of activities was
 25 detected, suggesting that excision of *Vader* may not always be precise.

Table 2

<u>Strain</u>	<u>% Nitrate Reductase Activity Compared to Wild-Type</u>
UVK143f (wild-type)	100
<i>niaD</i> 436 (<i>niaD</i> mutant)	ND ¹
Revertants of <i>niaD</i> 436:	
1	34.7
2	42.8
3	27.7
4	3.5
5	ND ¹

¹ Activity non-detectable

-- 16 --

Strain	% Nitrate Reductase Activity Compared to Wild-Type
6	47.4
7	90.4
8	9.8
9	25.4
10	28.9
11	38.2
12	6.9
13	71.7
14	71.7
15	49.7

Example 2

Cloning of a Vader Element

To determine if an insertion sequence was located within the *niaD* gene, two primers were synthesized. The first primer, *niaD*1 (SEQ ID NO:8), corresponded to position 142-165 of the *niaD* gene, and *niaD*2 (SEQ ID NO:9) corresponded to position 2738-2715 of the *niaD* gene. Genomic DNA was isolated from 14 unstable *niaD* mutants. This genomic DNA served as a template for the PCR primers. PCR reaction products with 4 *niaD* mutants (410, 436, 587 and 392) revealed an approximately 440 bp insertion (Vader) in the *niaD* gene.

For Southern blot analysis, genomic DNA isolated from the wild-type and four *niaD* mutants (410, 436, 587 and 392) was digested with BglII. The probe used was a Sall digestion fragment of the 500 bp PCR product generated using the *niaD*1 (SEQ ID NO:8) and *niaD*2 (SEQ ID NO:9) oligomeric probes. The probe hybridized to a 2.5 kb fragment with wild-type DNA (lane 5, Fig. 1). In the case of the *niaD* mutants 410 (lane 1, Fig. 1), 436 (lane 3, Fig. 1) and 392 (lane 4, Fig. 1), the probe hybridized to a 2.9 kb fragment. These results indicate that these three *niaD* mutants contain an approximately 440 bp insertion. Interestingly, with the mutant *niaD*587, the probe hybridized to both a 2.5 kb and 2.9 kb fragment, although mycelium had been grown in the experiment in the presence of KClO₃ to favor growth of the *niaD* mutant and not revertant cells, the detection of two hybridizable sequences indicated that in some cells Vader had been excised from the *niaD* gene.

The approximate location of the insertion was determined in each of the four unstable *niaD* mutants by restriction mapping analysis. The location of the insertion in each of the four mutants examined is shown in Fig. 2. All four mutants had an approximately 440 bp insertion located at different sites within the *niaD* gene.

-- 17 --

Example 3

Determination of Vader Copy Number

To determine the Vader copy number a 236 bp Scal-XbaI internal fragment of Vader-2 (cloned from the mutant *niaD*436) was hybridized to EcoRV cleaved genomic DNA. There is only one EcoRV site within the Vader transposon. Southern blot analysis indicated that there are approximately fifteen copies of Vader sequences in the genome of *A. niger* var. *awamori*. (Fig. 4). The Vader sequences were integrated at identical genomic locations in the three *niaD* mutants, 410, 436 and 587. However, in the *niaD*392 mutant, Vader sequences were located in five different locations compared to the three *niaD* mutants examined. This result was somewhat surprising considering that all four *niaD* mutants were isolated from the same strain, but provides good evidence for the high mobility of the Vader element in this strain. When a proprietary *A. niger* glucoamylase production strain (ETC #2663) was also examined, approximately 15 hybridization signals could be detected. Although some of the hybridization patterns appeared to be identical, clear differences could be seen between *A. niger* var. *awamori* and *A. niger*.

Example 4

Isolation of Vader in Other Fungal Species

In an attempt to determine if this transposable element was found in other filamentous fungi, genomic Southern blot analysis was performed using the 236 bp fragment (XbaI-ScaI) of Vader sequence as per Example 3, as a probe (Fig. 5). Two strains of *A. nidulans* were obtained from Fungal Genetics Stock Center (FGSC), FGSC #A691, a nitrate reductase structural gene mutant (*niaD*15), and FGSC #A237, a tryptophan-requiring mutant (*trpC*801). No hybridization signals could be visualized with strain A691, and a single strong hybridization signal could be detected with strain A237. These results support the notion that the lack of success in cloning transposable elements from laboratory strains of *A. nidulans* is due to low copy number or absence. Similarly, only one hybridization signal could be detected in *A. foetidus* and *A. phoenicis*, while two hybridization signals were detected in *A. cinnamomeus*. No hybridizations could be detected in *A. wentii* and *A. versicolor*. In addition, no hybridization signals could be detected with *Humicola grisea* var. *thermoidea*, *Neurospora crassa* and *Trichoderma reesei* (results not shown). These results indicate that the Vader element is most commonly found in *A. niger* var. *awamori* and *A. niger*.

-- 18 --

Example 5Excision of the Vader Element

Part of the *niaD* gene from *niaD436* containing the Vader element was amplified using PCR. The PCR amplification resulted in the expected 1200 bp fragment of the Vader element flanked by *niaD* sequences and a shorter 800 bp fragment resulting from the excision event. Sequencing of the shorter fragment indicated that the Vader element had excised precisely. However, when several revertants of *niaD436* and *niaD410* were assayed for their nitrate reductase activity (18), a spectrum of activities was detected, suggesting that excision of the Vader element may not always be precise (results not shown).

Example 6Isolation of Tan1

The previously isolated Vader element, although mobile, did not have an ORF encoding transposase activity presumed to be required for excision (22). This observation led to a search for a transposase-encoding larger element, thus an oligomer corresponding to the Vader inverted repeat was synthesized and used for PCR amplification of the genomic *A. niger* var. *awamori* DNA. The PCR amplification resulted in the generation of three DNA fragments: the 0.4 kb Vader element, as expected, and fragments of 1.9 kb and 2.3 kb in length.

Both of the larger PCR-generated fragments were sequenced and the sequences were identical with an exception that the 2.3 kb fragment had an additional 400 bp at the 3' end. Surprisingly this additional sequence at the 3' end was a Vader element, which differed only by a few nucleotides from the previously isolated Vader. The 5' end sequence, shared by both of the 1.9 kb and 2.3 kb fragments, had a single ORF (1668 bp) coding for a protein of 555 amino acids flanked by inverted repeats (IRs). Thus, the 1.9 kb fragment, devoid of the Vader element, had an organization of IR-ORF-IR. The larger 2.3 kb fragment had a unique organization, IR-ORF-IR-IR-Vader-IR, with a total of four inverted repeats (Figs. 9 and 11). In this larger element the two central inverted repeats, side by side, potentially form a tight hairpin structure, and despite many sequencing attempts with varying conditions, we were unable to determine the sequence between the two inverted repeats. However, the overall length of the PCR product, as determined by electrophoresis, corresponded to the size of the sequence shown in Fig. 11, suggesting that the two central contiguous IRs are not separated by a large segment of DNA.

Due to the organization of the 1.9 kb and 2.3 kb fragments, it was believed that the 1.9 kb fragment could have arisen in PCR from a partial amplification of the 2.3 kb

-- 19 --

fragment if the 3' IR-primer had annealed to the first central IR instead of the IR in the end of the Vader element. Southern analysis was conducted in order to determine if the 1.9 kb element existed in the genome without the associated Vader element, or whether it was a PCR-artifact derived from a partial amplification of the 2.3 kb element. The two probes used in Southern analysis corresponded to the internal sequence of Vader and to the carboxyterminal part of the ORF (Fig. 9). The genomic DNA from *A. niger* var. *awamori* *niaD* mutants and UVK143f were digested with *EcoRI*, which cuts once in the coding region of the ORF upstream from the ORF-probe and does not cut Vader. The Southern analysis showed numerous bands for the Vader element (Fig. 10), similar to previous Southern analyses (22). However, only one fragment lit up with the probe corresponding to the ORF and a fragment of the same size (1.6 kb) was recognized by the Vader probe (Fig. 10). It was concluded that the actual element in the genome was the 2.3 kb fragment and that the shorter 1.9 kb had only been a PCR-artifact. The isolated 2.3 kb fragment was designated as Tan1.

A genomic clone of the Tan1 element (2.3 kb) was isolated from a partial genomic library. Restriction enzymes, which were shown not to have any recognition sites in the PCR-amplified Tan1, were used separately and in combinations in Southern analysis of the genomic DNA. A double digestion with *BglII* and *XhoI* resulted in a relatively short, 4.5 kb, fragment which hybridized with the ORF-specific probe (data not shown). Genomic DNA fragments cleaved by *BglII* and *XhoI* and between 4 kb and 5 kb in size were cloned into pSP73 (Promega). The correct clone containing the Tan1 element was isolated by colony hybridization using the ORF-specific probe. Differences between the sequences of the genomic clone and the PCR-generated Tan1 were minor, even for the flanking IRs which were almost identical even though in the PCR-generated Tan1 the IRs were derived from the Vader IRs (PCR primers). It was seen from the genomic clone of Tan1 that immediately outside of the terminal IRs there were TA-dinucleotides, suggesting a TA target site and its duplication upon insertion. Sequence of the Tan1 genomic clone is shown in Figs. 11A and 11B [SEQ ID NO:13 (DNA) and SEQ ID NO:14 (amino acid)].

Example 7

Insertional Inactivation/Gene Tagging

Vader was cloned by insertional inactivation of the target gene *niaD*, which encodes nitrate reductase. The target sequence for integration of Vader is TA, a sequence which must be very common in the genome of fungi. Nitrate reductase mutants cannot grow on nitrate and inconsequence are resistant to the toxic analog of nitrate, KClO_3 .

-- 20 --

It is possible that one of the reasons heterologous protein production in fungi is lower than that of homologously produced protein using the same promoter is that the heterologous protein is being degraded by the cell. If there are genes whose products are responsible for degrading/sequestering foreign protein, it would be advantageous to inactivate those genes. In order to achieve this, a strain is constructed using gene disruption, which lacks the Tan1 gene. Such strain is then used to transform and express a heterologous protein such as the mammalian chymosin protein. It would be advantageous if the activity of such genes could be visualized or selected for on petri dishes. For example chymosin produced in *A. niger* results in a halo of clearing around a colony grown on skim milk. (See US Patent 5,364,770, the disclosure of which is incorporated herein by reference.)

Having transformed the strain with a construct comprising the desired heterologous protein or polypeptide, one would transform the strain a second time with Vader and Tan1 appropriately modified for gene tagging purposes.

The transformants are then plated on medium which can be used to visualize heterologous protein production, such as skim milk plates in the case of chymosin.

The plates are then screened for increased halo size, which is the result of inactivation of a gene whose product limits foreign protein production.

The inactivated gene can be cloned using the transposon sequences as a marker for cloning strategies. (See generally (19).)

Example 8

Elevation of Gene Expression Using Transposons

A reason that heterologous protein production is lower than expected in fungi is presumed to be that genes essential for foreign (heterologous) gene production are NOT expressed at sufficiently high levels in the fungi.

In order to overcome this problem, utilizing the transposable element(s) of the present invention, a strain is constructed in which the native Tan1 gene is inactivated by gene disruption.

This strain is used to express a heterologous protein whose expression can be easily visualized, such as chymosin (US Patent 5,364,770). A second transformation is made with Vader and Tan1, appropriately modified for gene tagging purposes. The internal sequence of Vader is replaced by a promoter sequence. One of the many integration events possible will be the integration of this promoter carrying Vader element into 5' to a gene beneficial to heterologous protein (e.g., chymosin) expression or secretion. Upon insertion, this beneficial gene is activated and such integrant colonies can be screened for,

-- 21 --

e.g., increased halo size (chymosin). The activated gene can be cloned using the transposon sequences as a marker for cloning strategies.

References

1. Schectman, M.G. (1987) *Mol. Cell. Biol.* **7**:3168-3177
2. Kinsey, J.A. & Hebler, J. (1989) *Proc. Natl. Acad. Sci. USA* **86**:1929-1933
3. Kinsey, J.A. (1993) *Proc. Natl. Acad. Sci. USA* **90**:9384-9387
4. Cambareri, E.B., Helber, J. & Kinsey, J.A. (1994) *Mol. Gen. Genet.* **242**:658-665
5. McHale, M.T., Roberts, I.N., Noble, S.M., Beaumont, C., Whitehead, M.P., Seth, D. & Oliver, R.P. (1992) *Mol. Gen. Genet.* **233**:337-347
6. Daboussi, M.J., Langin, T. & Brygoo, Y. (1992) *Mol. Gen. Genet.* **232**:12-16
7. Cove, D.J. (1976) *Heredity* **36**:191-203
8. Daboussi, M.J. & Langin, T. (1994) *Genetica* **93**:49-59
9. Lebrum, M.-H. Chumley, F. & Valent, B. (1994) *Fungal Genetics News Letter* **41A**:52
10. Kachroo, P., Leong, S.A. & Chattoo, B.B. (1994) *Mol. Gen. Genet.* **245**:339-348
11. Rowlands, R.T. & Turner, G. (1973) *Mol. Gen. Genet.* **126**:201-216
12. Sambrook, J., Fritsch, E.F. & Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Plainview, NY, pp. 1.11-1.85
13. Timberlake, W.E. & Barnard, E.C. (1981) *Cell* **26**:29-37
14. Yanisch-Peron, C., Vieira, J. & Messing, J. (1985) *Gene* **33**:103-119
15. Meseselson, M. & Yuan, R. (1968) *Nature* **217**:1110-1114
16. Charlesworth, B., Snlegowski, P. & Stephan, W. (1994) *Nature* **371**:215-220.
17. Fedoroff, N.V., Furtek, D.B. & Nelson, O.E. (1984) *Proc. Natl Acad. Sci. USA* **81**:3829-3835
18. Dunn-Coleman, N.S., Tomsch, A.D. & Garrett, R.H. (1981) *Molec. Gen. Getet.* **182**:234-239
19. Walden, R. & Schell, J. (1994) *Agro-Food-Industry-Hi-Tech*, Nov/Dec:9-12
20. Gems, D.H., Johnstone, I.L. & Clutterbuck, A.J. (1991) *Gene* **98**:61-67
21. Dunn-Coleman, N.S., Bloebaum P., Berka, R.M., Bodie, E., Robinson, N., Armstrong, G., Ward M., Przetak, M., Carter, G.L., LaCost, R., Wilson, L.J., Kodama, K.H., Baliu, E.F., Bower, B., Lamsa, M. & Heinsohn, H. (1991) "Commercial levels of chymosin production by *Aspergillus*," *Bio/Technology* **9**:976-981
22. Amutan, M., Nyyssonen, E., Stubbs, J., Diaz-Torres, M.R. & Dunn-Coleman, N. (1996) "Identification and cloning of a mobile transposon from *Aspergillus niger* var. *awamori*," *Curr. Genet.* **29**:468-473

23. Bancroft, I., Bhatt, A., Sjodin, C., Scofield, S., Jones, J. & Dean, C. (1992) *Mol. Gen. Genet.* **233**:449-461
24. Bancroft, I. & Dean, C. (1993) *Mol. Gen. Genet.* **240**:65-67
25. Long, D., Martin, M., Sundberg, E., Swinburne, J., Puangsomlee, P. & Coapland, G. (1993) *Proc. Natl. Acad. Sci.* **90**:10370-10374
26. Berka et al. (1990) *Gene* **86**:153-162
27. Ochman, H., Gerber, A.S. & Hart, D.L. (1988) *Genetics* **120**:621-623
28. Williams, J.F. (1989) *Biotechniques* **7**:762-769
29. Arnold, C. & Hodgson, I.J. (1991) *PCR Methods Appl.* **1**:39-42
30. Jones, D.H. & Winistorfer, S.C. (1993) *PCR Methods Appl.* **2**:197-203
31. Bruijn, F.J. & Lupski, J.R. (1984) *Gene* **27**:131-149
32. Brown, T. *Current Protocols in Molecular Biology*, Supplements 21, 24, 26 and 29
33. Minetoki, T., Gomi, K., Kitamoto, K., Kumagai, C., Tamura, G. (1995) "Nucleotide sequence and expression of alpha-glucosidase-encoding gene (*agdA*) from *aspergillus oryzae*," *Biosci. Biotechnol. Biochem.* **59**:1516-1521

-- 24 --

Sequence Listing

(1) GENERAL INFORMATION:

(i) APPLICANT: Amutan, Maria
Dunn-Coleman, Nigel
Nyyssonen, Eini M.

(ii) TITLE OF INVENTION: Identification of and Cloning a Mobile
Transposon from Aspergillus

(iii) NUMBER OF SEQUENCES: 17

(iv) CORRESPONDENCE ADDRESS:

(A) NAME: Genencor International, Inc.
(B) STREET: 925 Page Mill Road
(C) CITY: Palo Alto
(D) STATE: CA
(E) COUNTRY: USA
(F) POSTAL CODE (ZIP): 94304

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER: US
(B) FILING DATE: August 16, 1996
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Horn, Margaret A.
(B) REGISTRATION NUMBER: 33,401
(C) REFERENCE/DOCKET NUMBER: GC270-2

(xi) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: (415) 846-7536
(B) TELEFAX: (415) 845-6504

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 45 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

ACGTAATCAA CGGTCGGGCG GGCCACACGG TCAGGCGGGC CACCC

45

(2) INFORMATION FOR SEQ ID NO: 2:

-- 25 --

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 44 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

GATGGCCCGC CTGACCGTGT GGCCGCCCCG ACCGTTGATT ACGT 44

(2) INFORMATION FOR SEQ ID NO: 3:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 437 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

ACGTAATCAA CGGTCGAACG GGCCACACGG TCAGGCGGGC CATCCTGAAA TCCCATATAA 60
AAGATGTCTT GGGGATTCTA TTATATATCA ACCAGTACTA CTTCTATGAA GCTCTAACTT 120
TG TAGATAGT TATATATATA AGAATAAGTA TTCCATGAAT TTTTCAGATT TTAGAATTTT 180
TACTTTGATA ATGAAACCAG ATTCTTATAT AAAACATATA AATACAGATA TTGTAATATG 240
ATAAGTCCAT AAGTAAAAGT ATATTCATTT TTAGAAGGTA TATAGATATT ATTTATATTA 300
TTTAAAATCT ATATAGAAGA AATCTAATTC TTCTAGACCT GGATGGTAGA GATATATTAT 360
GTTTAAAAAG ATATCTTTTG TATAGTATTA CCAGATGGCC CGCCTGACCG TGTGGCCCGT 420
CCGACCGTTG ATTACGT 437

(2) INFORMATION FOR SEQ ID NO: 4:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 44 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

ACGTAATCAA CGGTCGAACG GGCCACACGG TCAGGCGGGC CATC 44

(2) INFORMATION FOR SEQ ID NO: 5:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 44 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

-- 26 --

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

GATGGCCCGC CTGACCGTGT GGCCCGTCCG ACCGTTGATT ACGT

44

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 2325 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

ACGTAATCAA CGGTCGGACG GGCCACACGG TCAGGCGGGC CATCCCTTCG AAAACACCAC	60
CTTGAATCAC CTACCCGAGG CTTTTCACCC ACCACAAATG CCACCAAAAG CATCTATCCC	120
ATCAAAATCG CAGGTGGAGC AGGAAGGCAG GATTCTTCTT GCCATTGAAG CTATTGAGAA	180
AGGCCAAATC ACTAGTATTC GTGAAGCAGC GCGTGTTCAT GACGTCGCTC GAACTACTCT	240
CCAGGCTCGA TTATCTGGAC GTGTTTTTCG TAAAAATATG ACCAACGCAC GTCAAAAATT	300
GTCAAATAAT GAAGAGGAAT CGCTTGTTAA ATGGATCCTA TCTCTAGATA AGCGAGGAGC	360
AAGCCCCCGG CCACTTGATA TCAGAGATAT GGCTAATTTG ATTATCTCTA AACGAGGTTA	420
TTCAACTGTT GAACAAGTAG GCATCAACTG GGCTTATAGC TTTGTAAAC GCCACGAATC	480
CCTACGAATC CGATTTGCTA GACGACTCAA CTATCCAAGA GCTAAAATGG AGGATCCTGA	540
AGTTATAAAA GACTGGTTCC AACGCGTACA GGAAGTTATT CAAGAGTACG GGATCTCATC	600
AGATGATATA TACAATTTTC ATGAAACAGG GTTTGCTATG GGAATGATTG CTACATATAA	660
AGTAGTAACT AGTTCCAGG GGGCAGGTCG GCCGTCCCTA GTTCAACCAG GGAATCGGGA	720
ATGGGTCACT CCAATTGAGT GTATTCGCTC TAATGGAGAG GTTCTACCTT CGACCCTGAT	780
CTTTAAAGGC AAAACACATC TAAAGGCATG GTATGAAGGT CAATCTATTC CTCCTACCTG	840
GAGATTTGAA GTCAGTGATA ATGGTTGGAC TACTGATAAA ATTGGACTTC GATGGCTTCC	900
AAAACACTTC ATTCCCTTGA TTAGAGGCAA ATCAGTAGGC AAATATAGCC TCCTAGTCCT	960
CGATGGCCAC GGTAGTCATT TGACACCTGA ATTCGACCAA TCCTGTGCTG AAAATGAGGT	1020
TATACCTATT TGTATGCCAG CTCATTCGTC CCATCTACTT CAGCCTCTTG ATGTTGGTTG	1080
TTTTAGTGTG CTAAACGCA CGTACGGAGG CATGGTTCCC AAGCAGATGC AATACGGCCG	1140
CAATCATATC GACAAGCTTG ACTTCTTAGA GGTCTATCCT AAAGCTCACC AGTGTGCTTT	1200
ATCAAAGTCG AATATAATCA GTGGTTTTAG AGCAACAGGT CTTGTTCCCTC TAGATCCTGA	1260
TCAAGTGCTT TCTCGACTCC ATATTCGCTT GAAACACCA CCAACCCCGG ATAGCCAGTC	1320
AAGTGGCTCA GTGCTTCAAA CACCACATAA TATAAACAC CTTTGGAGC ATCCAAAATC	1380
AGTGAACGC CTACTTCGGA AACGGCAAGC AAGTCCAAT TCACCTACAA ACTCTACACT	1440

-- 27 --

```

ACGTCAGCTT CTCAAAGGGT GTGAACTAGC AATAACAAAC TCAATCATAC TGGCTAAGGA      1500
GAATGCGGAA TTACGTGCTA GCCATGAAAA GCAACTACCA AAGAGGAAGC GTTCAAGGAA      1560
GCAGGTGATC TATACAGAAG GCACTACCGT TGAAGAGGCC CAGAGAGCTA TACAGGAAGT      1620
GGAAGAGGTG CAGAATGATG AAGATATTGA GGTGAACCC CAATCTCAAT ATACGGAGAC      1680
CCCCTCGCGC GCGCCTCCAC GCTGCAGTAA TTGCTTCAAT ATAGGCCACC GACGTACACA      1740
GTGTTCTAAA CCACCTACTA ATTAGTTAGA TAGCTGTTTT TACAAGCATT TATGTTGATT      1800
TAGAGGCCTC ATTTGGATCA TATCGGGTAA TCCTACCGGG AGATGGCCCG CCTGACCGTG      1860
TGGCCCCGCC GACCGTTGAT TACGTNNNNN ACGTAATCAA CGGTCGGACG GGCCCCCGG      1920
TCCGGCGGGC CATCTGGTAA TACTATACCA AAGATATCTT TTAAACATA ATATATCTCT      1980
ACCATCCAGG TCTAGGAGAA TTAGATTTCT TCTATATAGA TTTAAATAA TATAAATAAT      2040
ATCTATATAC CTTCTAAAAA TGAATATACT TTTACTTATG GACTTATCAT ATTACAATAT      2100
CTGTATTTAT ATGTATTATA TAAGAATCTG GTTTCATTAT CAAAGTAAAA ATTCTAAAAT      2160
CTGAAAAATT CATGGAATAC TTATTCTTAT ATATATAACT ATCTACAAAG TTAGAGCTTC      2220
ATAGAAGTAG TACTGGTTGA TATATAATAG AATCCCCAAG ACATCTTTTA TATGGGATTT      2280
CAGGATGGCC GCCGACCGTG TGGCCCGTCC GACCGTTGAT TACGT                        2325

```

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 555 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

```

Met Pro Pro Lys Ala Ser Ile Pro Ser Lys Ser Gln Val Glu Gln Glu
1           5           10           15

Gly Arg Ile Leu Leu Ala Ile Glu Ala Ile Gln Lys Gly Gln Ile Thr
20           25           30

Ser Ile Arg Glu Ala Ala Arg Val Tyr Asp Val Ala Arg Thr Thr Leu
35           40           45

Gln Ala Arg Leu Ser Gly Arg Val Phe Ala Lys Asn Met Thr Asn Ala
50           55           60

Arg Gln Lys Leu Ser Asn Asn Glu Glu Glu Ser Leu Val Lys Trp Ile
65           70           75           80

Leu Ser Leu Asp Lys Arg Gly Ala Ser Pro Arg Pro Leu Asp Ile Arg
85           90           95

Asp Met Ala Asn Leu Ile Ile Ser Lys Arg Gly Tyr Ser Thr Val Glu
100          105          110

```

-- 28 --

Gln Val Gly Ile Asn Trp Ala Tyr Ser Phe Val Lys Arg His Glu Ser
 115 120 125
 Leu Arg Thr Arg Phe Ala Arg Arg Leu Asn Tyr Pro Arg Ala Lys Met
 130 135 140
 Glu Asp Pro Glu Val Ile Lys Asp Trp Phe Gln Arg Val Gln Glu Val
 145 150 155 160
 Ile Gln Glu Tyr Gly Ile Ser Ser Asp Asp Ile Tyr Asn Phe Asp Glu
 165 170 175
 Thr Gly Phe Ala Met Gly Met Ile Ala Thr Tyr Lys Val Val Thr Ser
 180 185 190
 Ser Gln Arg Ala Gly Arg Pro Ser Leu Val Gln Pro Gly Asn Arg Glu
 195 200 205
 Trp Val Thr Pro Ile Glu Cys Ile Arg Ser Asn Gly Glu Val Leu Pro
 210 215 220
 Ser Thr Leu Ile Phe Lys Gly Lys Thr His Leu Lys Ala Trp Tyr Glu
 225 230 235 240
 Gly Gln Ser Ile Pro Pro Thr Trp Arg Phe Glu Val Ser Asp Asn Gly
 245 250 255
 Trp Thr Thr Asp Lys Ile Gly Leu Arg Trp Leu Pro Lys His Phe Ile
 260 265 270
 Pro Leu Ile Arg Gly Lys Ser Val Gly Lys Tyr Ser Leu Leu Val Leu
 275 280 285
 Asp Gly His Gly Ser His Leu Thr Pro Glu Phe Asp Gln Ser Cys Ala
 290 295 300
 Glu Asn Glu Val Ile Pro Ile Cys Met Pro Ala His Ser Ser His Leu
 305 310 315 320
 Leu Gln Pro Leu Asp Val Gly Cys Phe Ser Val Leu Lys Arg Thr Tyr
 325 330 335
 Gly Gly Met Val Pro Lys Gln Met Gln Tyr Gly Arg Asn His Ile Asp
 340 345 350
 Lys Leu Asp Phe Leu Glu Val Tyr Pro Lys Ala His Gln Cys Ala Leu
 355 360 365
 Ser Lys Ser Asn Ile Ile Ser Gly Phe Arg Ala Thr Gly Leu Val Pro
 370 375 380
 Leu Asp Pro Asp Gln Val Leu Ser Arg Leu His Ile Arg Leu Lys Thr
 385 390 395 400
 Pro Pro Thr Pro Asp Ser Gln Ser Ser Gly Ser Val Leu Gln Thr Pro
 405 410 415
 His Asn Ile Lys His Leu Leu Glu His Pro Lys Ser Val Glu Arg Leu
 420 425 430
 Leu Arg Lys Arg Gln Ala Ser Pro Thr Ser Pro Thr Asn Ser Thr Leu
 435 440 445

-- 29 --

Arg Gln Leu Leu Lys Gly Cys Glu Leu Ala Ile Thr Asn Ser Ile Ile
 450 455 460
 Leu Ala Lys Glu Asn Ala Glu Leu Arg Ala Ser His Glu Lys Gln Leu
 465 470 475 480
 Pro Lys Arg Lys Arg Ser Arg Lys Gln Val Ile Tyr Thr Glu Gly Thr
 485 490 495
 Thr Val Glu Glu Ala Gln Arg Ala Ile Gln Glu Val Glu Glu Val Gln
 500 505 510
 Asn Asp Glu Asp Ile Glu Val Glu Pro Gln Ser Gln Tyr Thr Glu Thr
 515 520 525
 Pro Ser Arg Ala Pro Pro Arg Cys Ser Asn Cys Phe Asn Ile Gly His
 530 535 540
 Arg Arg Thr Gln Cys Ser Lys Pro Pro Thr Asn
 545 550 555

(2) INFORMATION FOR SEQ ID NO: 8:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 23 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

CCAACCGAGT CCTCAGTATA GAC

23

(2) INFORMATION FOR SEQ ID NO: 9:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 24 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

CAACGCTTCA TAGGCGTCCA GATC

24

(2) INFORMATION FOR SEQ ID NO: 10:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 54 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

ATATGAATTC ACGTAATCAA CGGTCGGACG GGCCACACGG TCAGGCGGGC CATC

54

-- 30 --

(2) INFORMATION FOR SEQ ID NO: 11:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 30 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

ATATGAATTC CTTCTTGACT TCCCCGGAAC 30

(2) INFORMATION FOR SEQ ID NO: 12:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 30 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

ATATAAGCTT GTCACTGGAC GACATTTTCAG 30

(2) INFORMATION FOR SEQ ID NO: 13:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 2329 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

ACGTAATCAA CGGTCGGGCG GGCCACACGG TCAGGCGGGC CACCCCTTCG AAAACACCAC 60
CTTGAATCAC CTACCCGAGG CTTTTCACAC ACCACAAATG CCACCAAAG CATCTATCCC 120
ATCAAAATCG CAGGTGGAGC GGGAAGGCAG GATTCTTCTT GCCATTGAAG CTATTGAGAA 180
AGGCCAAATC ACTAGTATTC GTGAAGCAGC GCGTGTTTAT GACGTCGCTC GAACTACTCT 240
CCAGGCTCGA TTATCTGGAC GTGTTTTTCG TAAAAATATG ACCAACGCAC GTCAAAAATT 300
GTCAAATAAT GAAGAGGAAT CGCTTGTTAA ATGGATCCTA TCTCTAGATA AGCGAGGAGC 360
AAGCCCCCGG CCACTTGATA TCAGAGATAT GGCTAATTTG ATTATCTCTA AACGAGGTTA 420
TTCAACTGTT GAACAAGTAG GCATCAACTG GGCTTATAGC TTTGTAAAC GCCACGAATC 480
CCTACGAACT CGATTGCTA GACGACTCAA CTATCAAAGA GCTAAAATGG AGGATCCTGA 540
AGTTATAAAA GACTGGTTCA AACGCGTACA GGAAGTTATT CAAGAGTACG GGATCTCATC 600
AGATGATATA TACAATTTTCG ATGAAACAGG GTTTGCTATG GGAATGATTG CTACATATAA 660

-- 31 --

AGTAGTAACT	AGTTCCCAGA	GGGCAGGTCG	GCCGTCCCTA	GTTCAACCAG	GGAATCGGGA	720
ATGGGTCACT	GCAATTGAGT	GTATTGCTC	TAATGGAGAG	GTTCTACCTT	CGACCCTGAT	780
CTTTAAAGGC	AAAACACATC	TAAAGGCATG	GTATGAAGGT	CAATCTATTC	CTCCTACCTG	840
GAGATTTGAA	GTCAGTGATA	ATGGTTGGAC	TACTGATAAA	ATTGGACTTC	GATGGCTTCA	900
AAAACACTTC	ATTCCTTGA	TTAGAGGCAA	ATCAGTAGGC	AAATATAGCC	TCCTAGTCCT	960
CGATGGCCAC	GGTAGTCATT	TGACACCTGA	ATTCGACCAA	TCCTGTGCTG	AAAATGAGGT	1020
TATACCTATT	TGTATGCCTG	CTCATTCGTC	CCATCTACTT	CAGCCTCTTG	ATGTTGGTTG	1080
TTTTAGTGTG	CTTAAACGCA	CGTACGGAGG	CATGGTTCAA	AAGCAGATGC	AATACGGCCG	1140
CAATCATATC	GACAAGCTTG	ACTTCTTAGA	GGTCTATCCT	AAAGCTCACC	AGTGTGCTTT	1200
ATCAAAGTCG	AATATAATCA	GTGGTTTTAG	AGCAACAGGT	CTTGTTCCCTC	TAGATCCTGA	1260
TCAAGTGCTT	TCTCGACTCC	ATATTGCTT	GAAAACACCA	CCAACCCCGG	ATAGCCAGTC	1320
AAGTGGCTCA	GTGCTTCAAA	CACCACATAA	TATAAAACAC	CTTTTGAAGC	ATCCAAAATC	1380
AGTGGAACGC	CTACTTCGGA	AACGGCAAGC	AAGTCCAAC	TCACCTACAA	ACTCTACACT	1440
ACGTCAGCTT	CTCAAAGGGT	GTGAACTAGC	AATAACAAAC	TCAATCATAC	TGGCTAAGGA	1500
GAATGCGGAA	TTACGTGCTA	GCCATGAAAA	GCAACTACCA	AAGAGGAAGC	GTTCAAGGAA	1560
GCAGGTGATC	TATACAGAAG	GCACTACCGT	TGAAGAGGCC	CAGAGAGCTA	TACAGGAAGT	1620
GGAAGAGGTG	CAGAATGATG	AAGATATTGA	GGTTGAACCC	CAATCTCAAT	ATACGGAGAC	1680
CCCCTCGCGC	GCGCCTCCAC	GCTGCAGTAA	TTGCTTCAAT	ATAGGCCACC	GACGTACACA	1740
GTGTTCTAAA	CCACCTACTA	ATTAGTTAGA	TAGCTGTTTT	TACAAGCATT	TATGTTGATT	1800
TAGAGGCCTC	ATTTTGATCA	TATCGGGTAA	TCCTACCGAG	AGATGGCCCG	CCTGACCGTG	1860
TGGCCCGCCC	GACCGTTGAT	TACGTNNNNN	ACGTAATCAA	CGGTCCGACG	GGCCCCCGG	1920
TCCGGCGGGC	CATCTGGTAA	TACTATACAA	AAGATATCTT	TTTAAACATA	ATATATCTCT	1980
ACCATCCAGG	TCTAGGAGAA	TTAGATTTCT	TCTATATAGA	TTTTAAATAA	TATAAATAAT	2040
ATCTATATAC	CTTCTAAAAA	TGAATATACT	TTTACTTATG	GACTTATCAT	ATTACAATAT	2100
CTGTATTTAT	ATGTATTATA	TAAGAATCTG	GTTTCATTAT	CAAAGTAAAA	ATTCTAAAAA	2160
TCTGAAAAAT	TCATGGAATA	CTTATTCTTA	TATATATAAA	CTATCTACAA	AGTTAGAGCT	2220
TCATAGAAGT	AGTACTGGTT	GATATATAAT	AGAATCAAAA	AGACATCTTT	TATATGGGAT	2280
TTCAGGATGG	CCGCGCTGAC	CGTGTGGCCC	GTCGACCGT	TGATTACGT		2329

(2) INFORMATION FOR SEQ ID NO: 14:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 555 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

-- 32 --

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

Met	Pro	Pro	Lys	Ala	Ser	Ile	Pro	Ser	Lys	Ser	Gln	Val	Glu	Arg	Glu	1	5	10	15
Gly	Arg	Ile	Leu	Leu	Ala	Ile	Glu	Ala	Ile	Arg	Lys	Gly	Gln	Ile	Thr	20	25	30	
Ser	Ile	Arg	Glu	Ala	Ala	Arg	Val	Tyr	Asp	Val	Ala	Arg	Thr	Thr	Leu	35	40	45	
Gln	Ala	Arg	Leu	Ser	Gly	Arg	Val	Phe	Ala	Lys	Asn	Met	Thr	Asn	Ala	50	55	60	
Arg	Gln	Lys	Leu	Ser	Asn	Asn	Glu	Glu	Glu	Ser	Leu	Val	Lys	Trp	Ile	65	70	75	80
Leu	Ser	Leu	Asp	Lys	Arg	Gly	Ala	Ser	Pro	Arg	Pro	Leu	Asp	Ile	Arg	85	90	95	
Asp	Met	Ala	Asn	Leu	Ile	Ile	Ser	Lys	Arg	Gly	Tyr	Ser	Thr	Val	Glu	100	105	110	
Gln	Val	Gly	Ile	Asn	Trp	Ala	Tyr	Ser	Phe	Val	Lys	Arg	His	Glu	Ser	115	120	125	
Leu	Arg	Thr	Arg	Phe	Ala	Arg	Arg	Leu	Asn	Tyr	Gln	Arg	Ala	Lys	Met	130	135	140	
Glu	Asp	Pro	Glu	Val	Ile	Lys	Asp	Trp	Phe	Lys	Arg	Val	Gln	Glu	Val	145	150	155	160
Ile	Gln	Glu	Tyr	Gly	Ile	Ser	Ser	Asp	Asp	Ile	Tyr	Asn	Phe	Asp	Glu	165	170	175	
Thr	Gly	Phe	Ala	Met	Gly	Met	Ile	Ala	Thr	Tyr	Lys	Val	Val	Thr	Ser	180	185	190	
Ser	Gln	Arg	Ala	Gly	Arg	Pro	Ser	Leu	Val	Gln	Pro	Gly	Asn	Arg	Glu	195	200	205	
Trp	Val	Thr	Ala	Ile	Glu	Cys	Ile	Arg	Ser	Asn	Gly	Glu	Val	Leu	Pro	210	215	220	
Ser	Thr	Leu	Ile	Phe	Lys	Gly	Lys	Thr	His	Leu	Lys	Ala	Trp	Tyr	Glu	225	230	235	240
Gly	Gln	Ser	Ile	Pro	Pro	Thr	Trp	Arg	Phe	Glu	Val	Ser	Asp	Asn	Gly	245	250	255	
Trp	Thr	Thr	Asp	Lys	Ile	Gly	Leu	Arg	Trp	Leu	Gln	Lys	His	Phe	Ile	260	265	270	
Pro	Leu	Ile	Arg	Gly	Lys	Ser	Val	Gly	Lys	Tyr	Ser	Leu	Leu	Val	Leu	275	280	285	
Asp	Gly	His	Gly	Ser	His	Leu	Thr	Pro	Glu	Phe	Asp	Gln	Ser	Cys	Ala	290	295	300	

-- 33 --

Glu Asn Glu Val Ile Pro Ile Cys Met Pro Ala His Ser Ser His Leu
 305 310 315 320
 Leu Gln Pro Leu Asp Val Gly Cys Phe Ser Val Leu Lys Arg Thr Tyr
 325 330 335
 Gly Gly Met Val Gln Lys Gln Met Gln Tyr Gly Arg Asn His Ile Asp
 340 345 350
 Lys Leu Asp Phe Leu Glu Val Tyr Pro Lys Ala His Gln Cys Ala Leu
 355 360 365
 Ser Lys Ser Asn Ile Ile Ser Gly Phe Arg Ala Thr Gly Leu Val Pro
 370 375 380
 Leu Asp Pro Asp Gln Val Leu Ser Arg Leu His Ile Arg Leu Lys Thr
 385 390 395 400
 Pro Pro Thr Pro Asp Ser Gln Ser Ser Gly Ser Val Leu Gln Thr Pro
 405 410 415
 His Asn Ile Lys His Leu Leu Lys His Pro Lys Ser Val Glu Arg Leu
 420 425 430
 Leu Arg Lys Arg Gln Ala Ser Pro Thr Ser Pro Thr Asn Ser Thr Leu
 435 440 445
 Arg Gln Leu Leu Lys Gly Cys Glu Leu Ala Ile Thr Asn Ser Ile Ile
 450 455 460
 Leu Ala Lys Glu Asn Ala Glu Leu Arg Ala Ser His Glu Lys Gln Leu
 465 470 475 480
 Pro Lys Arg Lys Arg Ser Arg Lys Gln Val Ile Tyr Thr Glu Gly Thr
 485 490 495
 Thr Val Glu Glu Ala Gln Arg Ala Ile Gln Glu Val Glu Glu Val Gln
 500 505 510
 Asn Asp Glu Asp Ile Glu Val Glu Pro Gln Ser Gln Tyr Thr Glu Thr
 515 520 525
 Pro Ser Arg Ala Pro Pro Arg Cys Ser Asn Cys Phe Asn Ile Gly His
 530 535 540
 Arg Arg Thr Gln Cys Ser Lys Pro Pro Thr Asn
 545 550 555

(2) INFORMATION FOR SEQ ID NO: 15:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 44 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

ACGTAATCAA CGGTCGGACG GGCCCCCGG TCAGGCGGGC CATC

44

(2) INFORMATION FOR SEQ ID NO: 16:

-- 34 --

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 45 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

GGATGGCCCCG CCTGACCGTG TGGCCCGTTC GACCGTTGAT TACGT

45

(2) INFORMATION FOR SEQ ID NO: 17:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 44 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

ACGTAATCGG TAAGCGAGTT GCCCGCGCAA GCGAGTTGCC CACC

44

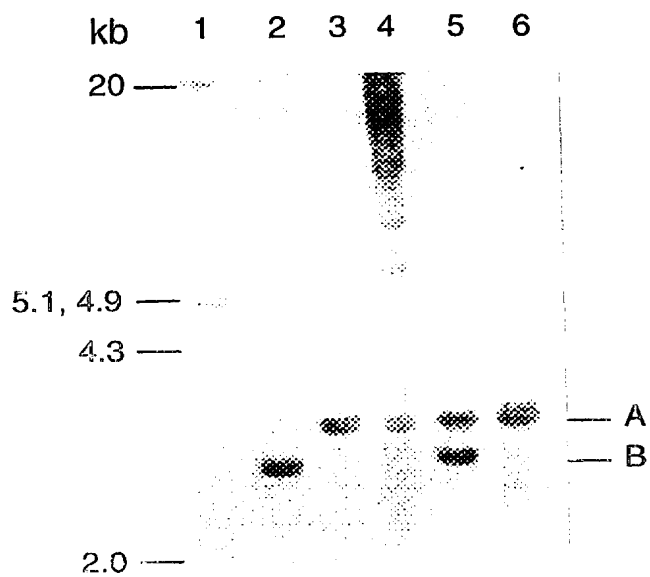
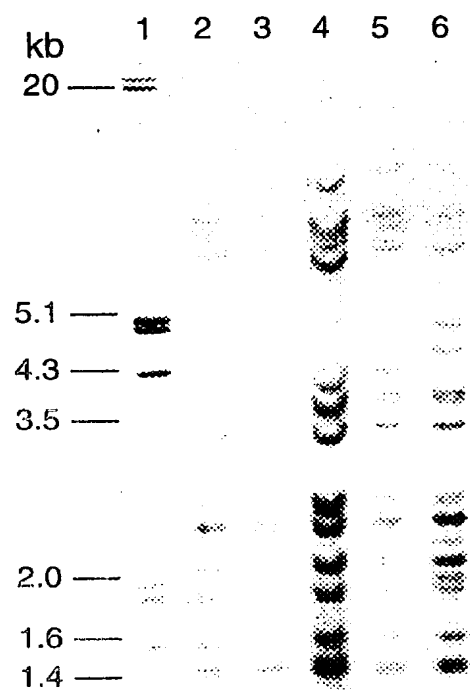
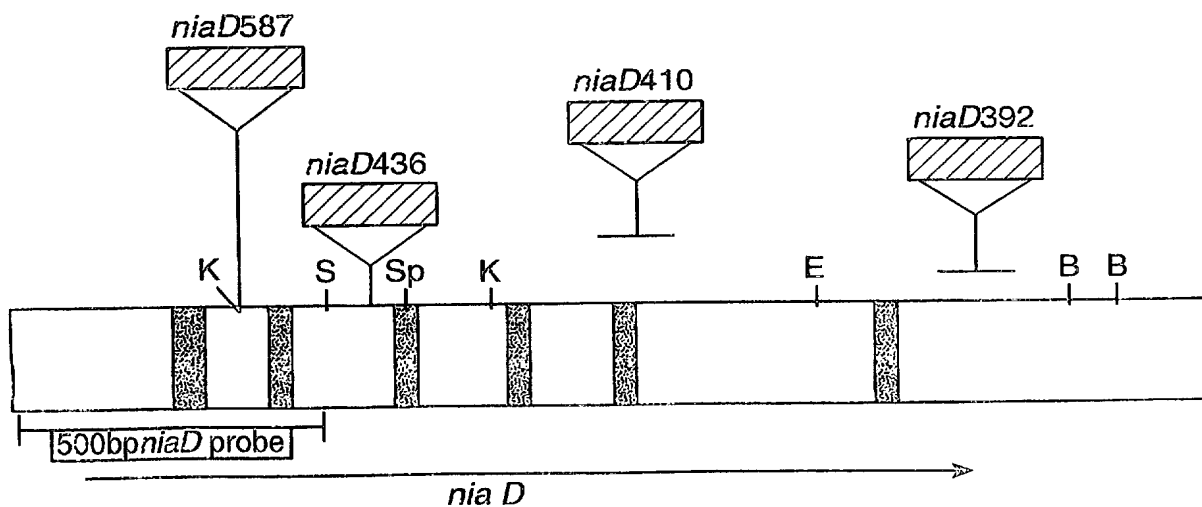
What is Claimed

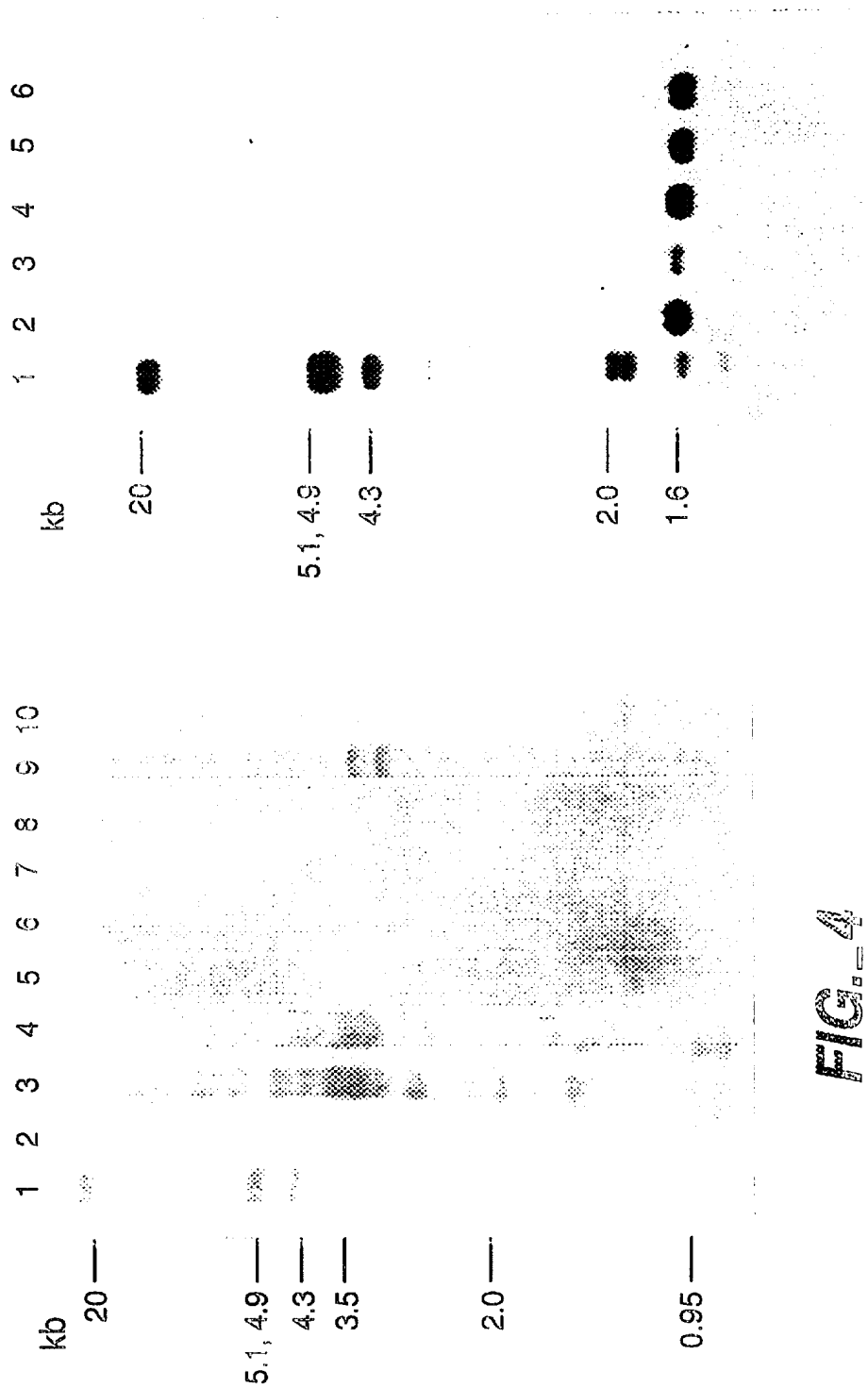
1. A transposable element isolated from *Aspergillus niger* var. *awamori* comprising a DNA fragment of about 2.3 kb which comprises SEQ ID NO: 1.
2. The transposable element of Claim 1 comprising the DNA sequence of SEQ ID NO:13 or variations thereof.
3. A fragment of the transposable element of Claim 1 comprising part or all of the DNA sequence selected from the group consisting of SEQ ID NOS:1 and 16 or variations thereof.
4. An isolated transposase coded for by the transposable element of Claim 1.
5. The transposase of Claim 4 comprising the amino acid sequence of SEQ ID NO:14.
6. A method of isolating a transposable element from a filamentous fungus, comprising the steps of:
 - (a) hybridizing fungal DNA under low stringency conditions to a probe, wherein the probe comprises part or all of one of the DNA fragments of Claim 3; and
 - (b) isolating fungal DNA which hybridizes to said probe.
7. The method of Claim 6 wherein the probe comprises an imperfect direct repeat within the DNA sequence selected from the group consisting of SEQ ID NOS:1 and 16.
8. A method of isolating a transposable element from a filamentous fungus genomic library, the method comprising probing said library with an ORF-specific probe and isolating DNA which hybridizes to said ORF-specific probe.
9. A method of isolating a transposable element from a filamentous fungi, the method comprising,
 - (a) subjecting fungal DNA to polymerase chain reaction amplification using part or all of one of the DNA fragments of Claim 3 as a primer, thereby generating amplified DNA sequences;
 - (b) isolating the amplified DNA sequences; and
 - (c) optionally identifying said amplified DNA sequence.
10. A transposable element isolatable by the method of Claim 6, 7, 8 or 9.

11. A method of isolating activation sequences comprising:
 - (a) inserting a marker gene within the inverted repeats of a transposable element of Claim 1 to form a modified marker gene having the structure IR-marker-IR;
 - (b) inserting the modified marker gene into a DNA target;
 - (c) selecting for expression of the modified marker; and
 - (d) isolating DNA upstream of said modified marker gene in said DNA target, which DNA upstream of said modified marker gene comprises an activation sequence driving expression of said modified marker gene.
12. A method for inactivating a gene in a host cell wherein said gene encodes a gene product, the method comprising:
 - (a) transforming a host cell with a genetic element to create a transformed host cell, wherein the genetic element comprises DNA for the gene and a transposable element of Claim 1 inserted within the DNA; and
 - (b) selecting for the transformed host cells which are deficient in the gene product.
13. A method for activating a desired gene in a host cell, the method comprising:
 - (a) inserting a regulatory gene within the inverted repeats of a transposable element of Claim 1 to form a modified regulatory gene having the structure IR-regulatory gene-IR;
 - (b) inserting the modified regulatory gene in DNA comprising the desired gene to form a DNA construct containing the modified regulatory gene upstream of said desired gene;
 - (c) transforming the host cell with the DNA construct; and
 - (d) selecting for transformants having enhanced expression of said desired gene.
14. A transposable element isolated from *Aspergillus oryzae* comprising a DNA fragment of at least about 1.2 kb.
15. The transposable element of Claim 14 comprising an inverted repeat DNA sequence of SEQ ID NO:17 or a variation thereof.
16. A fragment of the transposable element of 14 comprising part of all of the DNA sequence of SEQ ID NO:17 or a variation thereof.

17. A method of isolating a transposable element from a filamentous fungus, comprising the steps of:
- (a) hybridizing fungal DNA under low stringency conditions to a probe, wherein the probe comprises part or all of one of the DNA fragments of Claim 16; and
 - (b) isolating fungal DNA which hybridizes to said probe.
18. A method of isolating activation sequences comprising:
- (a) inserting a marker gene within the inverted repeats of a transposable element of Claim 10 to form a modified marker gene having the structure IR-marker-IR;
 - (b) inserting the modified marker gene into a DNA target;
 - (c) selecting for expression of the modified marker; and
 - (d) isolating DNA upstream of said modified marker gene in said DNA target, which DNA upstream of said modified marker gene comprises an activation sequence driving expression of said modified marker gene.
19. A method for inactivating a gene in a host cell wherein said gene encodes a gene product, the method comprising:
- (a) transforming a host cell with a genetic element to create a transformed host cell, wherein the genetic element comprises DNA for the gene and a transposable element of Claim 10 inserted within the DNA; and
 - (b) selecting for the transformed host cells which are deficient in the gene product.
20. A method for activating a desired gene in a host cell, the method comprising:
- (a) inserting a regulatory gene within the inverted repeats of a transposable element of Claim 10 to form a modified regulatory gene having the structure IR-regulatory gene-IR;
 - (b) inserting the modified regulatory gene in DNA comprising the desired gene to form a DNA construct containing the modified regulatory gene upstream of said desired gene;
 - (c) transforming the host cell with the DNA construct; and
 - (d) selecting for transformants having enhanced expression of said desired gene.

1 / 13

**FIG. 1****FIG. 3****FIG. 2**



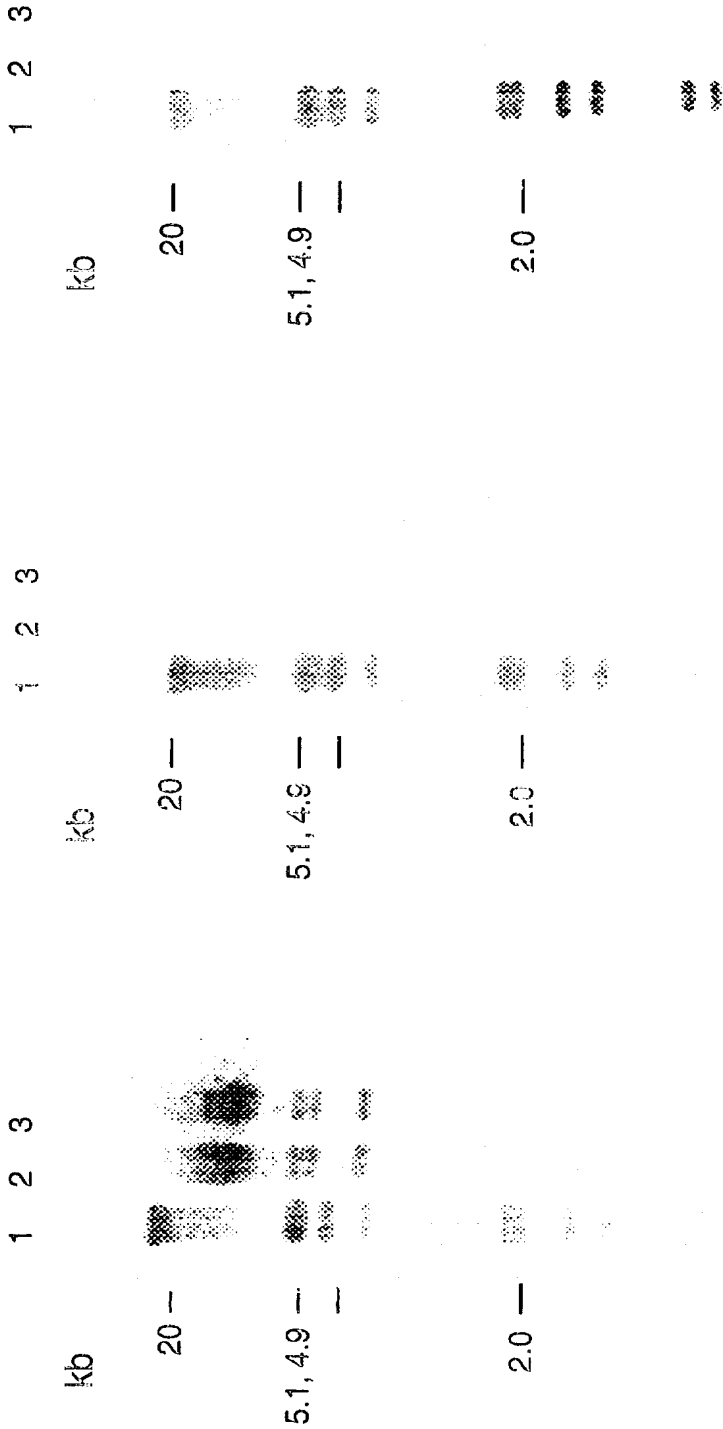


FIG._6C

FIG._6B

FIG._6A

4 / 13

tgtcgcacggctctctctggactggcccaatgatggcagatatcctacggagtgcga
agcctttgaggaaagccaag**ta**ACGTAATCAA CGGTCTG**A**ACG GGCCACACGG
TCAGGCGGGC CATCCTGAAA TCCCATATAA AAGATGTCTT GGGGATTCTA
TTATATATCA ACCAGTACTA CTTCTATGAA GCTCTAACTT TG TAGATAGT
TATATATATA AGAATAAGTA TTCCATGAAT TTTTCAGATT TTAGAATTTT
TACTTTGATA ATGAAACCAG ATTCTTATAT AAAACATATA AATACAGATA
TTGTAATATG ATAAGTCCAT AAGTAAAAGT ATATTCATTT TTAGAAGGTA
TATAGATATT ATTTATATTA TTTAAAATCT ATATAGAAGA AATCTAATTC
TTCTAGACCT GGATGGTAGA GATATATTAT GTTTAAAAAG ATATCTTTTG
TATAGTATTA CCAGATGGCC CGCCTGACCG TGTGGCCCGT **CCGACCGTTG**
ATTACGT**ta**tgtctgcatggaggaggctgataagctggtaagttaccttatcca
tccatgcatgcagtgccctga

FIG._7

5 / 13

1	ACGTAATCAA	CGGTCGGACG	GGCCACACGG	TCAGGCGGGC	CATCCCTTCG	AAAACACCAC	60
61	CTTGAATCAC	CTACCCGAGG	CTTTTCAACC	ACCACAAATG	CCACCAAAG	CATCTATCCC	120
			M	P	P	K A S I P	
121	ATCAAAATCG	CAGGTGGAGC	AGGAAGGCAG	GATTCCTTCTT	GCCATTGAAG	CTATTCAGAA	180
	S K S	Q V E Q	E G R	I L L	A I E A	I Q K	
181	AGGCCAAATC	ACTAGTATTC	GTGAAGCAGC	GCGTGTATTAT	GACGTCGCTC	GAACCTACTCT	240
	G Q I	T S I R	E A A	R V Y	D V A R	T T L	
241	CCAGGCTCGA	TTATCTGGAC	GTGTTTTCGC	TAAAAATATG	ACCAACGCAC	GTCAAAAATT	300
	Q A R	L S G R	V F A	K N M	T N A R	Q K L	
301	GTCAAAATAAT	GAAGAGGAAT	CGCTTGTTAA	ATGGATCCTA	TCTCTAGATA	AGCGAGGAGC	360
	S N N	E E E S	L V K	W I L	S L D K	R G A	
361	AAGCCCCCGG	CCACTTGATA	TCAGAGATAT	GGCTAATTTG	ATTATCTCTA	AACGAGGTTA	420
	S P R	P L D I	R D M	A N L	I I S K	R G Y	
421	TTCAACTGTT	GAACAAGTAG	GCATCAACTG	GGCTTATAGC	TTTGTTAAAC	GCCACGAATC	480
	S T V	E Q V G	I N W	A Y S	F V K R	H E S	
481	CCTACGAACT	CGATTTGCTA	GACGACTCAA	CTATCCAAGA	GCTAAAATGG	AGGATCCTGA	540
	L R T	R F A R	R L N	Y P R	A K M E	D P E	
541	AGTTATAAAA	GACTGGTTCC	AACGCGTACA	GGAAGTTATT	CAAGAGTACG	GGATCTCATC	600
	V I K	D W F Q	R V Q	E V I	Q E Y G	I S S	
601	AGATGATATA	TACAAATTTCG	ATGAAACAGG	GTTTGCTATG	GGAATGATTG	CTACATATAA	660
	D D I	Y N F D	E T G	F A M	G M I A	T Y K	

FIG._8A

6 / 13

661	AGTAGTAACT V V T	AGTTCCCAGA	GGCAGGTCG	GCCGTCCTA	GTTCAACCAG	GGAATCGGGA	720
721	ATGGGTCAC W V T	CCAATTGAGT	GTATTCGCTC	TAATGGAGAG	GTTCTACCTT	CGACCCCTGAT	780
781	CTTTAAAGGC	AAAACACATC	TAAAGGCATG	GTATGAAGGT	CAATCTATTTC	CTCCTACCTG	840
841	GAGATTTGAA	GTCAGTGATA	ATGGTTGGAC	TACTGATAAA	ATTGGACTTC	GATGGCTTCC	900
901	AAAACACTTC	ATTCCCTTGA	TTAGAGGCAA	ATCAGTAGGC	AAATATAGCC	TCCTAGTCCT	960
961	CGATGGCCAC	GGTAGTCATT	TGACACCTGA	ATTCGACCAA	TCCTGTGCTG	AAAATGAGGT	1020
1021	TATACCTATT	TGTATGCCAG	CTCATTCGTC	CCATCTACTT	CAGCCTCTTG	ATGTTGGTTG	1080
1081	TTTTAGTGTG	CTTAAACGCA	CGTACGGAGG	CATGGTTCCC	AAGCAGATGC	AATACGGCCG	1140
1141	CAATCATATC	GACAAGCTTG	ACTTCTTAGA	GGTCTATCCT	AAAGCTCACC	AGTGTGCTTT	1200
1201	ATCAAAGTCG	AATATAATCA	GTGGTTTTAG	AGCAACAGGT	CTTGTTCCCTC	TAGATCCTGA	1260

FIG._8B

7 / 13

1261	TCAAGTGCTT	TCTCGACTCC	ATATTGCTT	GAAAACACCA	CCAACCCCGG	ATAGCCAGTC	1320
	Q V L	S R L H	I R L	K T P	P T P D	S Q S	
1321	AAGTGGCTCA	GTGCTTCAAA	CACCACATAA	TATAAAACAC	CTTTTGAGC	ATCCAAAATC	1380
	S G S	V L Q T	P H N	I K H	L L E H	P K S	
1381	AGTGAACGC	CTACTTCGGA	AACGGCAAGC	AAGTCCAAC	TCACCTACAA	ACTCTACACT	1440
	V E R	L L R K	R Q A	S P T	S P T N	S T L	
1441	ACGTCAGCTT	CTCAAAGGGT	GTGAACTAGC	AATAACAAAC	TCAATCATA	TGGCTAAGGA	1500
	R Q L	L K G C	E L A	I T N	S I I L	A K E	
1501	GAATGCGGAA	TTACGTGCTA	GCCATGAAAA	GCAACTACCA	AAGAGGAAGC	GTTCAAGGAA	1560
	N A E	L R A S	H E K	Q L P	K R K R	S R K	
1561	GCAGGTGATC	TATACAGAAG	GCCTACCGT	TGAAGAGGCC	CAGAGAGCTA	TACAGGAAGT	1620
	Q V I	Y T E G	T T V	E E A	Q R A I	Q E V	
1621	GGAAGAGGTG	CAGAAATGATG	AAGATATTGA	GGTTGAAACCC	CAATCTCAAT	ATACGGAGAC	1680
	E E V	Q N D E	D I E	V E P	Q S Q Y	T E T	
1681	CCCCTCGCGC	GCGCCTCCAC	GCTGCAGTAA	TTGCTTCAAT	ATAGGCCACC	GACGTACACA	1740
	P S R	A P P R	C S N	C F N	I G H R	R T Q	
1741	GTGTTCTAAA	CCACCTACTA	ATTAGTTAGA	TAGCTGTTTT	TACAAGCATT	TATGTTGATT	1800
	C S K	P P T N	*				

FIG. 8C

8 / 13

1801	TAGAGGCCTC	ATTGGATCA	TATCGGGTAA	TCCTACCGGG	AGATGGCCCG	CCTGACCGTG	1860
1861	TGGCCCGCCC	GACCGTTGAT	TACGTNNNNN	ACGTAATCAA	CGGTCGGACG	GGCCCCCCGG	1915
1916	TCCGGCGGGC	CATCTGGTAA	TACTATACCA	AAGATATCTT	TTTAAACATA	ATATATCTCT	1975
1976	ACCATCCAGG	TCTAGGAGAA	TTAGATTCTT	TCTATATAGA	TTTAAATAAA	TATAAATAAT	2035
2036	ATCTATATAC	CTTCTAAAAA	TGAATATACT	TTTACTTATG	GACTTATCAT	ATTACAATAT	2095
2096	CTGTATTTAT	ATGTATTATA	TAAGAACTCTG	GTTTCATTAT	CAAAGTAAAA	ATTCTAAAAAT	2155
2156	CTGAAAAAAT	CATGGAATAC	TTATTCTTAT	ATATATAACT	ATCTACAAAG	TTAGAGCTTC	2215
2216	ATAGAAAGTAG	TACTGGTTGA	TATATAATAG	AATCCCCAAG	ACATCTTTTA	TATGGGATTT	2275
2276	CAGGATGGCC	GCCGACCGTG	TGGCCCGTCC	GACCGTTGAT	TACGT		2320

FIG._8D

9 / 13

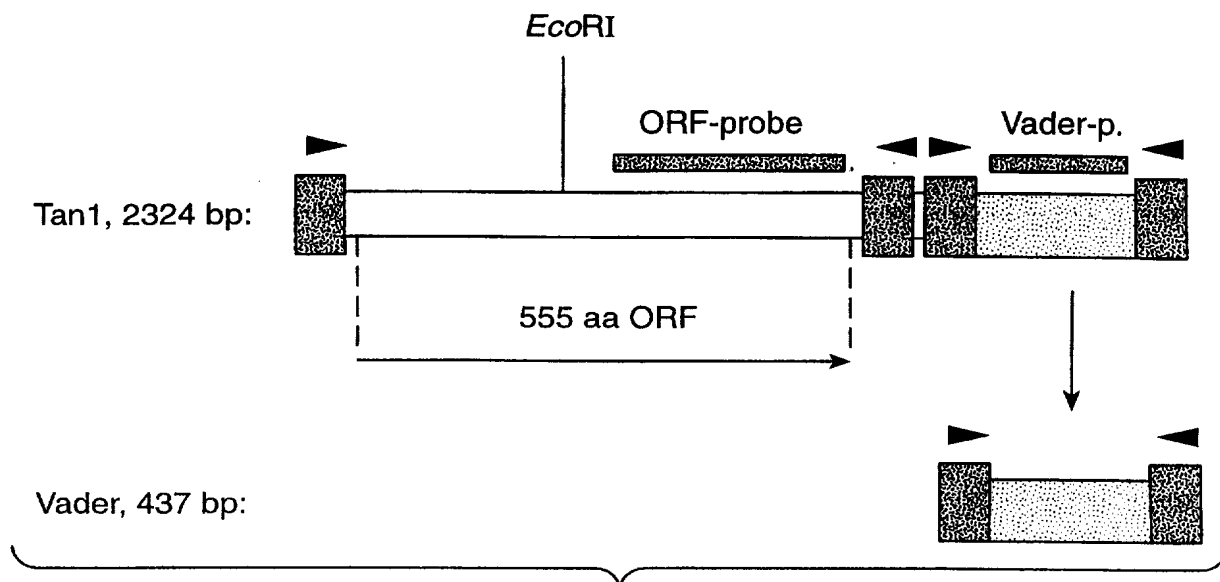


FIG._9

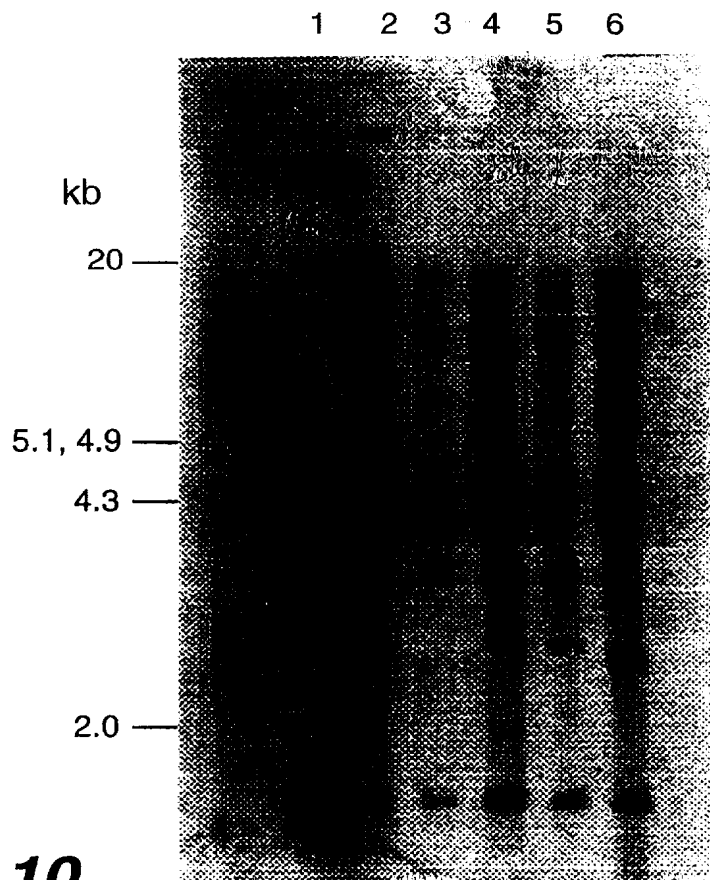


FIG._10

10 / 13

1	ACGTAATCAA	CGGTCGGGCG	GGCCACACGG	TCAGGGGGGC	CACCCCTTCG	AAAACACCAC	60													
61	CTTGAATCAC	CTACCCGAGG	CTTTTCAACC	ACCACAAATG	CCACCAAAAG	CATCTATCCC	120													
				M	P	P	K	A	S	I	P									
121	ATCAAAATCG	CAGGTGGAGC	GGGAAGGCAG	GATTCTTCTT	GCCATTGAAG	CTATTGAGAA	180													
	S	K	S	Q	V	E	R	E	G	R	I	L	L	A	I	E	A	I	R	K
181	AGGCCAAATC	ACTAGTATTC	GTGAAGCAGC	GCGTGTTTAT	GACGTCGCTC	GAACTACTCT	240													
	G	Q	I	T	S	I	R	E	A	A	R	V	Y	D	V	A	R	T	T	L
241	CCAGGCTCGA	TTATCTGGAC	GTGTTTTCGC	TAAAAATATG	ACCAACGCAC	GTCAAAAATT	300													
	Q	A	R	L	S	G	R	V	F	A	K	N	M	T	N	A	R	Q	K	L
301	GTCAAATAAT	GAAGAGGAAT	CGCTTGTTAA	ATGGATCCTA	TCTCTAGATA	AGCGAGGAGC	360													
	S	N	N	E	E	S	L	V	K	W	I	L	S	L	D	K	R	G	A	
361	AAGCCCCCGG	CCACTTGATA	TCAGAGATAT	GGCTAATTTG	ATTATCTCTA	AACGAGGTTA	420													
	S	P	R	P	L	D	I	R	D	M	A	N	L	I	I	S	K	R	G	Y
421	TTCAACTGTT	GAACAAGTAG	GCATCAACTG	GGCTTATAGC	TTTGTAAAC	GCCACGAATC	480													
	S	T	V	E	Q	V	G	I	N	W	A	Y	S	F	V	K	R	H	E	S
481	CCTACGAACT	CGATTTGCTA	GACGACTCAA	CTATCAAAGA	GCTAAAATGG	AGGATCCTGA	540													
	L	R	T	R	F	A	R	R	L	N	Y	Q	R	A	K	M	E	D	P	E
541	AGTTATAAAA	GACTGGTTCA	AACGCGTACA	GGAAGTTATT	CAAGAGTACG	GGATCTCATC	600													
	V	I	K	D	W	F	K	R	V	Q	E	V	I	Q	E	Y	G	I	S	S
601	AGATGATATA	TACAATTTTCG	ATGAAACAGG	GTTTGCTATG	GGAATGATTG	CTACATATAA	660													
	D	D	I	Y	N	F	D	E	T	G	F	A	M	G	M	I	A	T	Y	K

FIG._ 11A

11 / 13

661	AGTAGTAACT V V T S S Q R A G R P S L V Q P G	720	GGAATCGGGA N R E
721	ATGGGTCAC T GCAATTGAGT GTATTGCTC TAATGGAGAG GTTCTACCTT	780	CGACCCCTGAT T L I
781	CTTTAAAGGC AAAACACATC TAAAGGCATG GTATGAAGGT CAATCTATT	840	CTCCTACCTG P T W
841	GAGATTTGAA GTCAGTGATA ATGGTTGGAC TACTGATAAA ATTGGACTTC	900	GATGGCTTCA Q
901	AAAACACTTC ATTCCCTTGA TTAGAGGCAA ATCAGTAGGC AAATATAGCC	960	TCCTAGTCCT L V L
961	CGATGGCCAC GGTAGTCATT TGACACCTGA ATTCGACCAA TCCTGTGCTG	1020	AAAATGAGGT N E V
1021	TATACCTATT TGTATGCCCTG CTCATTGCTC CCATCTACTT CAGCCTCTTG	1080	ATGTTGGTTG C
1081	TTTTAGTGTG CTTAAACGCA CGTACGGAGG CATGGTTCAA AAGCAGATGC	1140	AATACGGCCG R
1141	CAATCATATC GACAAGCTTG ACTTCTTAGA GGTCTATCCT AAAGCTCACC	1200	AGTGTGCTTT L
1201	ATCAAAGTCG AATATAATCA GTGGTTT TAG AGCAACAGGT CTGTGTCCTC	1260	TAGATCCTGA D

FIG. 11B

12 / 13

1261	TCAAGTGCTT	TCCTCGACTCC	ATATTGCTT	GAAACACCA	CCAACCCCGG	ATAGCCAGTC	1320
	Q V L	S R L H	I R L	K T P	P T P D	S Q S	
1321	AAGTGGCTCA	GTGCTTCAAA	CACCACATAA	TATAAAACAC	CTTTTGAAGC	ATCCAAAATC	1380
	S G S	V L Q T	P H N	I K H	L L K H	P K S	
1381	AGTGAACGC	CTACTTCGGA	AACGGCAAGC	AAGTCCAAC	TCACCTACAA	ACTCTACACT	1440
	V E R	L L R K	R Q A	S P T	S P T N	S T L	
1441	ACGTCAGCTT	CTCAAAGGGT	GTGAACTAGC	AATAACAAAC	TCAATCATA	TGGCTAAGGA	1500
	R Q L	L K G C	E L A	I T N	S I I L	A K E	
1501	GAATGCGGAA	TTACGTGCTA	GCCATGAAA	GCAACTACCA	AAGAGGAAGC	GTTCAAGGAA	1560
	N A E	L R A S	H E K	Q L P	K R K R	S R K	
1561	GCAGGTGATC	TATACAGAAG	GCACTACCGT	TGAAGAGGCC	CAGAGAGCTA	TACAGGAAAGT	1620
	Q V I	Y T E G	T T V	E E A	Q R A I	Q E V	
1621	GGAAGAGGTG	CAGAATGATG	AAGATATTGA	GGTTGAACCC	CAATCTCAAT	ATACGGAGAC	1680
	E E V	Q N D E	D I E	V E P	Q S Q Y	T E T	
1681	CCCCTCGGC	GCGCCTCCAC	GCTGCAGTAA	TTGCTTCAAT	ATAGGCCACC	GACGTACACA	1740
	P S R	A P P R	C S N	C F N	I G H R	R T Q	
1741	GTGTTCTAAA	CCACCTACTA	ATTAGTTAGA	TAGCTGTTTT	TACAAGCAAT	TATGTTGATT	1800
	C S K	P P T N	*				

FIG._11C

13 / 13

1801	TAGAGGCCTC	ATTTTGATCA	TATCGGGTAA	TCCTACCGAG	AGATGGCCCG	CCTGACCGTG	1860
1861	TGGCCCGCCC	GACCGTTGAT	TACGT???	ACGTAATCAA	CGGTCGGACG	GGCCCCCCGG	1915
1916	<u>TCAGGCGGGC</u>	<u>CATCTGGTAA</u>	<u>TACTATACAA</u>	<u>AAGATATCTT</u>	<u>TTTAAACATA</u>	<u>ATATATCTCT</u>	1975
1976	ACCATCCAGG	TCTAGGAGAA	TTAGATTCT	TCTATATAGA	TTTTAAATAA	TATAAATAAT	2035
2036	ATCTATATAC	CTTCTAAAAA	TGAATATACT	TTTACTTATG	GACTTATCAT	ATTACAATAT	2095
2096	CTGTATTAT	ATGTATTATA	TAAGAACTCG	GTTTCATTAT	CAAAGTAAAA	ATTCTAAAAA	2155
2156	TCTGAAAAAT	TCATGGAATA	CTTATTCTTA	TATATATAAA	CTATCTACAA	AGTTAGAGCT	2215
2216	TCATAGAAGT	AGTACTGGTT	GATATATAAT	AGAATCAAAA	AGACATCTTT	TATATGGGAT	2275
2276	TTCAGGATGG	CCCGCCCTGAC	CGTGTGGCCC	GTCGACCGT	TGATTACGT		2324

←-----↓-----↓

FIG._11D

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 97/14978

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/81 C12N9/22 C12Q1/68 C12N15/11

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C07K C12N C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>AMUTAN M ET AL: "IDENTIFICATION AND CLONING OF A MOBILE TRANSPOSON FROM ASPERGILLUS NIGER VAR. AWAMORI" CURRENT GENETICS, vol. 29, no. 5, 1 April 1996, pages 468-471. XP000576438 see abstract see page 470, right-hand column, paragraph 1 - page 471, right-hand column, paragraph 1 see page 472, left-hand column, paragraph 2 see page 473, left-hand column, paragraph 2 - last paragraph</p> <p style="text-align: center;">--- -/-</p>	<p>1,3, 6-10, 14-16</p>

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

5 December 1997

Date of mailing of the international search report

30/12/1997

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

Authorized officer

Montero Lopez, B

INTERNATIONAL SEARCH REPORT

Int: ional Application No

PCT/US 97/14978

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	GLAYZER, DIANNE C. ET AL: "The isolation of Ant1, a transposable element from <i>Aspergillus niger</i> " MOL. GEN. GENET. (1995), 249(4), 432-38 CODEN: MGGEAE; ISSN: 0026-8925, 1995, XP002049285	8,10
A	see abstract see page 434, right-hand column, paragraph 2 - page 437, left-hand column, paragraph 2	1-7,9, 11-20
X	MARK T. MCHALE ET AL.: "CfT-I: an LTR-retrotransposon in <i>Cladosporium fulvum</i> , a fungal pathogen of tomato" MOLECULAR AND GENERAL GENETICS, vol. 233, no. 3, June 1992, BERLIN DE, pages 337-347, XP002049286 cited in the application see abstract see page 338, left-hand column, paragraph 3 see page 338, right-hand column, last paragraph - page 339, left-hand column, paragraph 2	8,10
A	TOSHITAKA MINETOKI ET AL.: "Nucleotide sequence and expression of alpha-glucosidase-encoding gene (agdA) from <i>Aspergillus oryzae</i> " BIOSCIENCE, BIOTECHNOLOGY, AND BIOCHEMISTRY, vol. 59, no. 8, August 1995, pages 1516-1521, XP002049287 cited in the application see abstract; figure 2	1-5,10, 14-16
P,X	WO 96 29414 A (GENENCOR INT) 26 September 1996 see page 1, paragraph 1 see page 3, paragraph 3 - page 4, paragraph 2; figures 8-10 see page 7, paragraph 4 - page 8, paragraph 1; examples 2,4,6,7	1-4, 6-10,12, 14-17,19
	-/--	

INTERNATIONAL SEARCH REPORT

Int :ional Application No

PCT/US 97/14978

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	<p>E. NYSSÖNEN ET AL.: "The transposable element Tan1 of Aspergillus niger var. awamori, a new member of the Fot1 family" MOLECULAR AND GENERAL GENETICS, vol. 253, no. 1-2, 27 November 1996, BERLIN DE, pages 50-56, XP002049288 see abstract see page 51, right-hand column, last paragraph - page 53, right-hand column, paragraph 1 see page 55, left-hand column, paragraph 3 - right-hand column, paragraph 2 -----</p>	<p>1-10, 14-16</p>

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 97/14978

Patent document
cited in search report

Publication
date

Patent family
member(s)

Publication
date

WO 9629414 A

26-09-96

NONE